Summer November 2014

INFLUENCE OF LIPOTEICHOIC ACID ON LISTERIA MONOCYTOGENES BIOFILM FORMATION AND CELLULAR STRESS

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INFLUENCE OF LIPOTEICHOIC ACID ON *LISTERIA MONOCYTOGENES* BIOFILM FORMATION AND CELLULAR STRESS

A Dissertation Presented

by

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Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

SEPTEMBER 2014

DEPARTMENT OF FOOD SCIENCE

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DEDICATION

This is dedicated to my family;

my father Ateng Tirtajaya, who taught me that things can still go wrong no matter how much we prepared and planned for it not to happen,
my mother Shinta Sari Boentaran, who taught me what it means to be a strong and independent woman who knows what she wants and how to get there,
my sisters Meida and Meidie Tirtajaya, who showed me what sisters are all about.

And to my husband James Matthew Brooks, who showed me what it means to be loved, that we are stronger when we are together, and that even when nothing goes the way it should, it will eventually be alright.

ACKNOWLEDGEMENTS

People told me that getting a PhD is a difficult and exhausting journey, and it is not a journey that one should take alone. That being said, like other PhD graduates, I would not have succeeded without the help and support of many people, whom I am about to acknowledge on this page.

My family; my father Ateng Tirtajaya, my mother Shinta Sari Boentaran, my sisters Meida and Meidie Tirtajaya, for allowing to pursue my dream of getting this terminal degree, even at times when they did not understand why I wanted to do this, and with the consequences of not seeing me and not being with me as often as they would like. This is their accomplishment as much as mine.

My chair committee and my advisor; Dr. Lynne McLandsborough. I thank her for giving me the opportunity to work under her supervision, and be part of her lab at UMass Food Science department. I am grateful for her guidance, support, and most of all, her patience. When I started this project, not even knowing what magic a thermal cycler can do, she promised to get me through. And I am forever grateful to her for holding on to her words that I can finally get this degree. She taught me what it means to not be a quitter, to be persistent, and to believe that there is light at the end of the tunnel. She has made me a better student, a better researcher, and a better person with her advice, positive attitude, appreciation, and on-going support.

My other committee members; Dr. Ronald Labbe, Dr. Susan Leschine, and Dr. Yeonhwa Park, for their patience and their understanding, even when this project has to be delayed for nine months while I was away in South Carolina. I thank them for their questions, inputs and considerations to make this dissertation better in the end. I also thank Dr. Julian McClements for his generosity for allowing me to use the Zeta Master and the Nikon Confocal Microscope in his lab.

My lab group, both past and present; Dr. John Cotter, whom I consider a brother that I do not have, Dr. Yuhua Chang, for his help during the first few months of this project, Kyle Landry, for his wide trivia knowledge and the things that he shares about 'life', and Kaitlin Ewald, for her encouragement and her persistence in getting me back into the dating scene. Fujia Zhang, Elsina Hagan, Ejoke Ememu, Lufan Wang, and Dillon Murray; they taught me that there is more to life as a graduate student than just being at the lab doing research. And that there is more to talk about than merely publications and exams.

My favorite labmate, aka. Dr. Chanelle Adams. I have the privilege of knowing this wonderful and beautiful woman as we go through our PhD journeys together. Through our conversations over meals at the office, at the Blue Wall, while we were studying for our Lipid Chemistry exam, even across our lab benches as we did our dilutions and the Bunsen burners are on, her positive attitude about life, and her confidence taught me that beauty is about the inside shining out, and that I do not have to be alone as I go through this journey. I thank her for everything she has done for me; from giving me rides to South Amherst at nights or on a rainy day, to being in my wedding. I am blessed beyond measure to be able to call her not only my colleague and my labmate, but also my friend.

There are many people outside McLandsborough Lab that have been a tremendous help to me as I go through this journey. I thank Ruth Witkowsky for her help with chemicals and lab organizing over the years, especially when the 4th floor construction started this summer, and Jean Alamed for her help when I need to borrow chemicals and equipment. Also Iris Joye and Bicheg Woo, for helping me with the Nikon confocal microscope, to the point where I am no longer afraid of that instrument, and Cynthia Lopez Pena for her help with the Zeta Master instrument.

For the last few years I am grateful that throughout this journey I have met people who are willing to take me in and be my family while my biological family is halfway across the globe. I thank Dawn Murry, who graciously opens her house, not only to me but also

to my husband as we stay over the summer. Her kindness and her generousity in providing us a place to stay have been such a blessing and have made our lives so much easier this summer. I also thank Linda Goulet for her prayers and emotional support.

InterVarsity Christian Fellowship is the closest that I can have to having brothers and sisters. I especially thank Michael and Carol Greene, Stephen Oloo, Addison Mayberry, Keiko Konoeda, and Hannah Masse, for their love and support, for great discussions that goes well beyond Friday nights at Goodell.

The Faith-Science Integration discussion groups have been an important part of my journey as a PhD student. I thank Dr. Mike Foster, Dr. Richard Vachet, and Dr. Craig Nicolson, for being such an inspiration. Spending time with these wonderful scientists showed me that it is possible to be a good and faithful Christian, who believe in God, while being a good scientist and a good researcher at the same time.

I thank Alison Raskin and Alina Bahnean for their wonderful friendship. I thank them for the time we spent together; dinner at Bertucci's and Chili's, lunch at Panera, brunch at Stables, dinner at House of Terriyaki, even looking at wedding magazines at Barnes and Noble. I thank them even for showing interest on what a 'deletion mutant' is, and for making me laugh even when I had been hitting my head against the wall at the lab for weeks.

Last but not least, to my husband, who is the love my life, James Brooks. Thank you for your kindness, your confidence, your positive attitude, your patience, and your support, especially during these last hectic two months. You show me what it means to be loved and to be persistent, and I could not imagine going through these last couple of months without you by my side. You are God's greatest blessing to me, and I thank Him for sending you into my life at such a perfect time. Thank you for believing in me even when I could not find it in myself to know how to believe in myself. Now enjoy reading this from front to back, because this is also your accomplishment as much as it is mine.

ABSTRACT

THE INFLUENCE OF LIPOTEICHOIC ACID IN *LISTERIA MONOCYTOGENES*

BIOFILM FORMATION AND CELLULAR STRESS

SEPTEMBER 2014

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Lipoteichoic acid (LTA) is an important polymer on the surface of Gram-positive organisms, including the foodborne, biofilm-forming pathogen *Listeria monocytogenes*, and is connected to the cell membrane through a glycolipid backbone. Previous results have shown the importance of LTA in cell viability and biofilm formation. The purpose of this research is to further investigate the influence of LTA in *L. monocytogenes* biofilm formation and how the bacterium responds to environmental stress caused by antimicrobial exposures, and a combination of low temperature and high osmolarity. We created in frame deletion mutants along the operon *lmo-2555-lmo2554-lmo2553*, which have been shown to reduce the ability of *L. monocytogenes* to form biofilm. We were able to show that our mutant strain had less amount of LTA on their cell membrane and formed less biofilm both on a PVC well plate and drip flow reactor. Visualization of biofilm cells using confocal microscopy also showed that our mutant strain had thinner biofilm compared to its positive control and its complement strain. We believe that the reduction in biofilm formation was due to the ability of cells to attach prior to forming biofilms. Our mutant strains also had increased sensitivity toward anionic and cationic antimicrobials. The influence of LTA was more pronounced when cells were exposed to low temperature, where our mutant strain was not able to grow even after a 14-day incubation at 4^{o}C. Our

strains also showed different sensitivity to various humectants at lower temperature (4^{o}C and 20^{o}C), where glycerol was the least harsh, while sucrose was the harshest. The absence of LTA did not seem to influence the cells' tolerance on high osmolarity in the presence of NaCl. However, our mutant strain showed considerable difference in osmotolerance in the presence of sucrose. Further understanding of biofilm formation mechanism will eventually lead to a more intelligent design of *L. monocytogenes* biofilm removal in the food processing environment.

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CHAPTER I

INTRODUCTION

Listeria monocytogenes (*L. monocytogenes*) is a ubiquitous organism that has a wide range of pH and temperature growth. It can also cause a disease called listeriosis that has a 20% mortality rate. Immuno compromised people are highly at risk for listeriosis. Based on this, the federal government requires a zero tolerance for *L. monocytogenes* in ready-to-eat foods.

Lipoteichoic acid (LTA) is an important polymer on the surface of Gram positive organisms and is connected to the cell membrane through a glycolipid backbone. The structure of LTA varies from one organism to another, but often consists of a polyglycerol phosphate backbone chain which is linked to the cell membrane by a glycolipid anchor. Previous studies in different Gram positive organisms like *Staphylococcus aureus* and *Bacillus subtilis* have confirmed the importance of LTA in contributing to normal cell growth and division.

In *L. monocytogenes*, the glycolipid that links LTA to the cell membrane is Gal(α1-2)Glc(α1-3)-diacylglycerol (Gal-Glc-DAG) or Gal(α1-2)Ptd-6Glc(α1-3)DAG. Previous research has confirmed that 2 particular genes, namely *lmo2555* and *lmo2554*, which are part of *lmo2555-lmo2554-lmo2553* operon, are involved in the synthesis of this glycolipid. We observed a severely-reduced biofilm formation when transposon mutagenesis is performed along the *lmo2555-lmo2554-lmo2553* operon, which leads us to believe that *p*henotypically these genes appear to be critical for initial adhesion and biofilm formation.

The purpose of this research is to further investigate the function of the genes in this operon by creating in-frame deletion mutants of each gene. Characterization and

complementation of each deletion mutants will also be performed in order to further determine the function of this gene in *L. monocytogenes* biofilm formation.

CHAPTER II

LITERATURE REVIEW

2.1 *Listeria monocytogenes*

Listeria monocytogenes (*L. monocytogenes*) is a Gram positive, rod-shaped, pathogenic, motile and ubiquitous organism found in 17 and 37 species of bird and mammalian, respectively. It is commonly found in soil and water and on plant material, especially decaying plant material, which makes it the natural habitat of this organism (1). It belongs to the genus Listeria, with 5 other species; *L. ivanovii, L. innocua, L. seeligeri, L. welshimeri,* and *L. grayi.* Among these 5 species, only *L. monocytogenes* that has the ability to lyse red blood cells, a characteristic that makes it different from the other five species and makes it considered to be a human pathogen (2).

L. monocytogenes cause a disease called listeriosis, although approximately 10% of human population carries *L. monocytogenes* in their intestine without becoming sick (3). Listeriosis itself in many cases is not characterized by the presence of gastrointestinal symptoms, such as nausea, vomiting and diarrhea (4). But in some cases, listeriosis may be preceded by these symptoms (1). In healthy adults, listeriosis is likely not going to go further than mild gastrointestinal symptoms, but in immuno-compromised adults such as pregnant women, the elderly and people with AIDS, the infection may lead to meningitis and blood infection with 20% mortality rate. Other manifestation includes septicemia, encephalitis, and intrauterine or cervical infections in pregnant women, which may result in spontaneous abortion (1^{st} or 2^{nd} trimester) or stillbirth (1).

Foods that have been associated with listeriosis include raw milk, chese, ice cream, raw vegetables, raw meat sausages, raw and cooked poultry, raw meat, raw and cooked fish, even human breast milk (1, 2). The U.S. Department of Agriculture and U.S.

Food and Drug Administration established zero tolerance policies for *L. monocytogenes* in foods in 1989, and since then the food industry have launched efforts to reduce *Listeria* contamination. But this is proven to be challenging due to the ubiquitous nature of the organism. *L. monocytogenes* has the ability to grow even under refrigeration temperature. Most strains of *L. monocytogenes* require a minimum a_w 0.93 to grow, although most strains can still grow at a_w below 0.90 (2). In fish and fisheries products, *L. monocytogenes* has a minimum requirement of a_w as low as 0.92 and pH range 4.4 to 9.4, temperature range 0.4 to 45^{o}C and can tolerate 10% salt concentration (1).

2.2 Biofilm formation

Microorganisms can attach to surfaces and develop into biofilms, which are complex communities of microorganisms marked by the excretion of a protective and adhesive matrix. Biofilms are often characterized by an attachment to the surface of a solid material, heterogeneous structure and complex community interactions. In food processing environments, biofilm formation is unwanted because biofilms may contain foodborne pathogens and spoilage microorganisms that can lead to product contamination (5). Furthermore, in many cases biofilms are more resistant toward detergents and antibiotics. It is proven that hot water, chlorine, iodophors and most quaternary ammonium compounds are ineffective against them (6).

Most scientists believe that biofilm formation starts when bacteria sense environmental conditions that trigger the transition from living as planktonic cells to living attached to a surface, which vary among organisms (7). The first step is the formation of a conditioning layer (8). This step involves adsorption of inorganic or organic molecules to the surface of a solid material, which is later called the conditioning layer. In many cases, milk and meat proteins are important elements of this conditioning layer because they actually aid the adhesion of bacteria to the surface. But in a milk processing plant, milk

proteins, such as caseins, α-Lactalbumin and β-Lactoglobulin significantly reduced the attachment of bacteria to the surface (9). This might be due to the pI of the milk proteins. Caseins, α-Lactalbumin and β-Lactoglobulin have pI below 6 and therefore were negatively charged in milk (pH around 6.5). The negatively charged proteins and bacterial cells may account for the decrease in attachment. Studies have shown that in environments where nutrients were plentiful, the nutrients act as a bacterial primer that increases the ability of bacteria to attach to a surface (8).

As soon as a conditioning layer is formed, it is easy for cells to attach to the surface and form biofilm. Interactions between bacteria and solid surfaces in required in order forming a stable biofilm. This implies that an overall repulsive and attractive force between cells and surface significantly affects the formation of biofilm. Not only that a conditioning layer is important, but some scientists would argue that specific outer membrane adhesins are required for cells to attach as the following step of biofilm formation. In other words, a stable attachment is formed not solely dependent upon a conditioning layer (5, 8). The cells themselves play a role by genetically switching from planktonic to 'attachment' mode. This starts with the determination of their cell density, which is a process called quorum sensing triggered by a small, water soluble molecules. These molecules are known as autoinducers (7).

In Gram positive bacteria, like *Staphylococcus aureus,* these autoinducers are peptides recognized as thiolactones (10). In addition, some reports have shown that in Gram negative bacteria, such as *Pseudomonas aeruginosa*, although flagella and type IV pili were not necessary for initial attachment or biofilm formation, cell appendages do have a significant role in biofilm development (11), as well as a simple adhesin called Curli plays an important role in *E. coli* biofilm formation (12).

There are many factors that affect bacterial adhesion to a surface, including the pH and temperature of the contact surface, flow rate of fluid passing over the surface,

nutrient availability, length of time the bacteria is in contact with the surface, bacterial growth stage and surface hydrophobicity (5). Initially the bonds between the bacteria and EPS may not be strong and can easily be removed by flowing water. However, with time, these bonds are becoming stronger so that it is impossible to detach them without applying further and stronger physical force like scrubbing or scraping (8).

The final stage of a biofilm formation is bacterial growth and biofilm expansion. Once firmly attached to a surface, injured cells can repair, metabolize fatty acids and protein contained within the conditioning layer. They will later on grow and reproduce (8). As the bacteria grow and reproduce, they excrete greater amount of exopolymeric substances (EPS) that provides even greater protection around the cells. At this stage, simply passing sanitizer over the top of biofilms will not be adequate for removal.

2.3 Teichoic Acids (TA) and Lipoteichoic Acid (LTA)

The terminology ‘teichoic acid’ was first used to represent a group of polymers containing phosphate groups isolated exclusively from the walls of Gram positive bacteria. The earliest examples were polymers containing either glycerol phosphate or ribitol phosphate, in which repeating units are joined together through phosphodiester linkages. In many cases various glycosyls are attached glycosidically to the polyol residues, and D-alanyl substitutes are attached through labile ester linkages to the hydroxyl groups. The term ‘teichoic acid’ has since been extended to cover all bacterial cell wall capsular and membrane polymers that contain glycerol phosphate or ribitol phosphate residues (13), (14). However, it is also important to note that not all Gram positive bacteria have lipoteichoic acids, with *Actinomyces* and *Micrococcus* being two notable genera that lack of this molecule (14). They generally have similar anionic polymer that function very similarly to wall teichoic acids (15). For example, lipomannan is found in place of

lipoteichoic acid in *Micrococcus luteus*. Its polyanionic character is determined by succinyl groups esterified to the mannosyl residues, as reviewed by Neuhaus and Baddiley (16).

Teichoic acid can be divided into two major classes according to their location within the cellular framework. Wall teichoic acids has been shown to have a wide structural diversity in Gram positive organisms. Some of this diversity is determined by the presence and nature of the glycosyl substituents, namely D-alanyl esters, and their monomers. The monomers are joined by anionic phosphodiester linkages to form linear chains that contribute a major part (approximately 30 – 60%) of the cell wall and are linked covalently to the peptidoglycan. Membrane teichoic acids, however, possess glycerol phosphate chains that are covalently linked to the glycolipid molecules located within the cell membrane (13, 16-18).

Other than the different locations within the cellular framework, so far, several major distinctions have been established between wall and membrane teichoic acids. Cell wall teichoic acids are covalently-linked to peptidoglycan and may exhibit a wide range of glycerol phosphate and ribitol phosphate-containing structures now included in the teichoic acid definition and they are not present in all Gram-positive organisms. Their occurrence may also dependent upon the growth condition employed to the species. It has been proposed by Archibald, *et. al.* (17) that the chains of wall teichoic acid in *Bacillus subtilis* are arranged perpendicular to the surface of the wall. A similar organization was also observed in many strains of *Staphylococcus aureus* years later (19).

Membrane teichoic acids, however, are linked to the glycolipid moiety of the plasma membrane and are more of a characteristic component of Gram positive organisms (20) in which their occurrence is not as dependent upon growth conditions as the occurrence of cell wall teichoic acids. It was discovered that after series of membrane preparations, it is possible to obtain membrane teichoic acid with its glycolipid portion intact. This led to the use of the term ‘lipoteichoic acid’ for this group of polymers, as

suggested by Lambert, *et. al.* in 1977, (13) following Knox and Wicken who first introduced this terminology in 1973 (20).

Much later publications refer to 'lipoteichoic acid' as a polymer of repetitive 1,3-phosphodiester-linked glycerol-1-phosphate units with a glycolipid anchor (21). Unlike wall teichoic acid polymers, which have been studied extensively, membrane teichoic acids received considerably far less attention, possibly due to the difficulties entailed in their isolation and purification (13).

Many bacteria have both the wall and lipoteichoic acid systems, which are synthesized by distinct biochemical routes, even when the actual repeating unit is the same (22). In the case of *Bacillus subtilis*, for example, both the wall and lipoteichoic acid consists of poly (glycerol phosphate), or poly(groP). However, the wall and lipoteichoic acid are synthesized by separate pathways that are genetically distinct and different from one another (16, 22).

Even though wall and lipoteichoic acid in *Bacillus subtilis* have identical structures, apart from their linkage to peptidoglycan and cell membrane, respectively, it is fairly been assumed that the spatial distribution of the two polymers determines their specialized functions. In principle, this means that lipoteichoic acid remains closely connected to the cell membrane surface, whereas wall teichoic acid should migrate out through the wall as it matures (23).

2.4 Occurrence of lipoteichoic acid in Gram positive organisms

2.4.1 Location of lipoteichoic acid in the cell

For many years, it has been confirmed that membrane teichoic acids, or lipoteichoic acids, can be isolated from nearly all Gram positive bacteria (24). They were also referred to as 'intracellular' teichoic acids since they were not situated in the cell wall, but found in the interface of the cytoplasmic membrane and wall instead (13, 22).

However, in *Staphylococcus aureus*, Aasjord and Grov (25) were able to show that lipoteichoic acid is not only attached to the membrane, but also penetrates the wall. For organisms that do not contain wall teichoic acid, such as *Lactobacillus fermentum*, van Driel, *et. al.* (26) found that one portion of the lipoteichoic acid is exposed on the cell surface while a second portion is concentrated at the membrane. Lipoteichoic acid is also released from the cell surface during growth.

High speed centrifugation of the cell contents fraction of disrupted cells caused the intracellular teichoic acid to sediment together with the membrane fragments and ribosomes (27). Extraction from a variety of Gram positive organisms, namely lactobacilli, streptococci and bacilli, and gel chromatography showed lipoteichoic acids as high molecular weight micellar complexes analogous to the lipopolysaccharide micelles extracted from Gram negative organisms by similar procedures (28). However, the same methods of extraction seem to yield different result when performed on different bacteria. When *Streptococcus faecalis* was converted into protoplasts, the product of its membrane preparations contained most of the teichoic acid originally present in the cell (29). However, similar studies with *Bacillus megaterium* and various group D streptococci gave different results, where most of the lipoteichoic acid is being released into the supernatant when cells are converted into protoplasts (13, 30).

2.4.2 Lipoteichoic acid biosynthesis and structure

Lipoteichoic acid biosynthesis occurs via transfer of glycerolphosphate units from phosphatidylglycerol with the formation of elongated lipoteichoic acid and diaclglycerol, as reviewed by Neuhaus and Baddiley (16). The mechanism of elongation, the organization of this assembly system, and the attachment of the glycolipid anchor are not yet well understood.

On the other hand, comparative studies of lipoteichoic acid glycosylation in organism such as *Bacillus subtilis*, for example, have been able to illustrate aspects of structural diversity (31). Structural studies on different LTA (26, 32) have shown them to consist of 1→3 phopsphodiester linked chains of 25 to 30 glycerol phosphate residues substituted with glycosyl and D-alanyl ester groups (33). The lipid portion is a glycolipid identical to the free glycolipid of the cytoplsmic membrane, or in some cases a phosphatidyl or glycerophosphoryl derivative thereof, linked through a phosphodiester bond involving a sugar hydroxyl group of the glycolipid and the terminal glycerol phosphate residue of the polyglycerol phosphate chain, as reviewed by Lambert *et. al.* (13)

Other publications have stated that lipoteichoic acids are classified into three different types, as reviewed by Neuhaus and Baddiley (16) and previously reported by other groups (32, 34, 35). Type I lipoteichoic acids are macroamphiphiles composed of polyglycerol phosphates or poly(GroP) attached to C-6 of the nonreducing glucosyl of the glycolipid anchor. The glycolipid is Glc(β1-6)Glc(β1-3)(gentiobiosyl)diacyl-GroP in staphylococci, bacilli and streptococci (36, 37). Type II lipoteichoic acid has a –GalGal-GroP- repeating unit, while Type III lipoteichoic acid has a –Gal-GroP- repeating unit.

All types of lipoteichoic acids may have different constituents, but one that has been shown to highly affect the properties of lipoteichoic acids and furthermore affect the cell membrane is the D-alanine component, and the synthesis of D-alanyl-lipoteichoic acid, or D-alanyl-LTA requires four proteins that are encoded by the *dlt* operon, as reviewed by Neuhaus and Baddiley (16). The D-alanyl ester contents of both lipo and wall teichoic acid have been shown to be a function of the pH of the growth medium (17, 38), temperature (39), and high concentration of NaCl in the media (40, 41). For the latter, Koch, *et. al.* suggested that the high concentration of NaCl directly affects one of the enzymes involved in the synthesis of D-alanyl-LTA.

Although the structure and substitution of the glycolipid moiety may vary from one organism to another (28), lipoteichoic acids are clearly amphipathic molecules, in that each has long, polar glycerol phosphate chain linked to a small hydrophobic lipid portion of the lipid bilayer in the plasma membrane (33, 42-44). Magnesium (Mg^{2+}) ions also play an important role in this association. It has become clear that the concentration of Mg^{2+} present during formation of the protoplasts is an important factor that controls the association of lipoteichoic acid with the membrane (26, 45).

It has been mentioned previously in this Literature Review that the synthesis of D-alanyl-LTA in Gram positive organisms requires four proteins that are encoded by the *dlt* operon. In *Staphylococcus aureus* and *Staphylococcus xylosus*, inactivation of *dlt* by either random transposon or targeted mutagenesis results in in increased sensitivity of these bacteria to many antimicrobial cationic peptides (46). The hypothesis so far is that the enhanced sensitivity is correlated with the higher net polyanionic charge of the lipoteichoic acid in the mutant deficient of D-alanyl-ester. However, mutants that are deficient in the poly(GroP) moiety of lipoteichoic acid have not been successfully isolated (16). This may reflect either the essential role of lipoteichoic acid in growth or the fact that the mechanism of lipoteichoic acid assembly is not completely understood.

2.5 Physiological functions of lipoteichoic acids in Gram positive organisms

It is so far quite evident that lipoteichoic acid (LTA) have physiological functions and are indispensable for growth of Gram positive organisms. This is based on at least two supporting factors. The first factor is the widespread and the phenotypically invariant occurrence of LTA in the membranes of these organisms (13, 47). The second factor that supports this hypothesis is the fact that a substantial proportion of the metabolic activity in many Gram positive bacteria is directed towards synthesis of teichoic acid, thus it seems

reasonable to assume that these polymers have some role or function which is of significant value to the cell (45)

Studies so far have shown the importance of LTA in the physiology of cells, from acting as membrane carrier in the synthesis of wall teichoic acids (48, 49), as a scavenger or in interaction with divalent cations (50), or in the adherence of oral streptococci to dental surfaces (51), in addition to acting as a regulator or inhibitor of autocatalytic activity (52, 53), as reviewed by Meyer and Wouters (54). Some of the major functions will be discussed below. Since D-alanine is one of the major constituents of LTA (16), it is somewhat expected that the roles of LTA discussed below are highly dependent upon this particular component.

2.5.1 The role of lipoteichoic acid in cell viability

Wall teichoic acid was originally thought to be essential for physiological functions in Gram positive bacteria. This is based on the fact that deletions of gene affecting the later step in the pathway that leads to the synthesis of wall teichoic acid were proven to be lethal to *Bacillus subtilis* (55). However, a more recent result showed that the apparent lethality experienced by the cells is due to the accumulation of toxic intermediates. When the gene that encodes the first enzyme in the pathway is deleted, there was no accumulation of toxic intermediates, and the cells are viable, even though they are severely compromised. They still maintain their ability to divide, but lose their ability to maintain a rod shape, thus become swollen and almost look round (56). This shows that, at least in the case of *Bacillus subtilis*, wall teichoic acid system plays a special role in elongation, which also have been shown in previous publications (57) and later publications (58).

So far, the lipoteichoic acid system and the genes that encode the biochemical pathway are poorly understood, especially when it comes to its role in cell growth and

division. However, Grundling, *et. al.* (2007) was able to pinpoint the key enzyme that catalyzes the formation of poly(glycerol phosphate), or poly(GroP), which is a lipoteichoic acid component in *Staphylococcus aureus* (59). This enzyme is known as LTA synthase (LtaS). A homologue of this enzyme in *Bacillus subtilis* was shown by Schirner, *et. al.* to possess the same activity and its complementation to *ltaS* deletion mutant has proven to be successful in maintaining cell growth (23).

The same group (Schirner, 2009) also found that a deletion of three other *ltaS* paralogues in *Bacillus subtilis* disrupts not only cell division during the vegetative state, but also spore formation, thus revealing that lipoteichoic acid is somewhat essential for this purpose, although these genes contribute to the function of lipoteichoic acid differently. However, complete disruption of wall and lipoteichoic acid pathways is lethal to the bacteria, this revealing that teichoic acid synthesis is essential in *Bacillus subtilis* (23).

Despite the findings that wall and lipoteichoic acid systems in *Bacillus subtilis* contributes differently to cell viability (23, 56, 57), it has also been found that in other organisms, such as *Staphylococcus aureus*, wall and lipoteichoic acid have somewhat overlapping and partially redundant roles that are required for cell viability and various cell wall properties (60). Lipoteichoic acid seems to play crucial part in cell division, which makes it indispensable for cell viability, as reviewed by Weidenmaier, *et. al.* (61). It has been shown that *Staphylococcus aureus* mutants that are deficient in lipoteichoic acid has distorted cell shapes and division sited (59).

2.5.2 Lipoteichoic acid as a membrane carrier in wall teichoic acid synthesis

It is the original suggestion that the precursors for the synthesis of wall teichoic acids were the nucleotides cytidine diphosphate glycerol (CDP-glycerol) and cytidine

diphosphate ribitol (CDP-ribitol). This suggestion has also been confirmed by demonstration in the later years (62, 63).

Later, using *Staphylococcus aureus* as an example, Fiedler, *et. al.* prepared a soluble form of polyribitol phosphate synthase from the membranes by extraction with non-ionic surfactant Triton X-100. They reported that the synthesis of polymer from CDP-ribitol in the presence of enzyme is entirely dependent upon the addition of an acceptor macromolecule that has the properties of a membrane teichoic acid (64). A similar system, although far less characterized, has also been demonstrated in *Bacillus subtilis* (65).

So far, it has been reported that in *Staphylococcus aureus*, and probably in many other bacteria, the polyribitol phosphate chain finally becomes attached to the cell wall by means of a linkage unit that contains a linear chain of three glycerol phosphate residues interposed between the phosphate-terminal end of the polyribitol phosphate chain and a muramic acid residue in the peptidoglycan (66, 67). This leads to the discovery by Hancock and Baddiley in 1976 (68) that polyribitol phosphate attached to linkage unit was formed as a membrane-bound amphiphilic species before the teichoic acid became linked to the wall. Following this hypothesis, Hancock *et. al.* later reported that in the presence of CDP-glycerol and another component, namely UDP-*N*-acetylglucosamine, CDP-ribitol gives rise to polyglycerol phosphate derivatives that can be separated from polyribitol phosphate-lipoteichoic acid carrier by ion exchange chromatography (69). They later on proposed a biosynthetic scheme, in which completed polyribitol phosphate chains are transferred from lipoteichoic acid carrier to a membrane-bound form of the linkage unit, probably a lipid, before being incorporated into the cell wall.

Taking all of these discoveries together, it appears that lipoteichoic acid serves as a membrane-bound carrier, mediating the transfer of a water-soluble polyribitol phosphate chain to a membrane-bound lipid-glycerol phosphate unit to incorporate a polymer into the

cell wall. It is interesting, however, that CDP-glycerol does not seem to participate in the biosynthesis of lipoteichoic acid (13, 50).

2.5.3 Lipoteichoic acid and its interaction with divalent cations

The early suggestion that teichoic acid might participate in ion exchange reactions and influence the passage of ionic materials through the cell surface was based largely on a consideration of its ionic nature, and that polymers containing phosphate groups, including teichoic acids, bind cations due to their polyanionic character. (45, 50). In addition to that, divalent cations are bound more effectively than are monovalent cations. This leads to the hypothesis that one of the roles of teichoic acids in the bacterial envelope is to maintain the correct balance of divalent cations in that part of the cells.

Of all the different divalent and monovalent cations required by bacteria to maintain the state of normal growth, magnesium (Mg^{2+}) ions are known to be required for the stability of isolated cell membranes and for the activity of many membrane-bound enzymes. Therefore it is suggested that a major function of both wall teichoic acid and lipoteichoic acid might be to maintain a high concentration of Mg^{2+} ions in the region of the membrane (70).

A publication by Ellwood and Tempest (38) has shown that several Gram positive bacteria synthesize another negatively charged polymer, namely teichuronic acid, instead of wall teichoic acid when grown in media with moderate ionic strength under limited phosphate. Therefore these bacteria can survive without wall teichoic acids, although they require an alternative polymer with negative charge. It was also found that lipoteichoic acid was still present even when bacteria were grown under conditions which led to the synthesis of teichuronic acid into the wall, which indicated that even though wall teichoic acid can be functionally replaced by other anionic polymers, the presence of lipoteichoic acid is apparently essential for the cell to function properly. This may explain why

lipoteichoic acid is present in all Gram positive bacteria which have been examined so far, whereas not all these bacteria have wall teichoic acids (18).

Hughes, *et. al.* (71) has shown the ability of teichoic acid to maintain the optimum concentration of Mg^{2+} in the region of the cell membrane, and that Mg^{2+}-dependent enzymes in cytoplasmic membranes in *Bacillus licheniformis* fully activated the enzymes only when it is bound to lipoteichoic acids. When both free and bound Mg^{2+} were present, the bound ions interacted preferentially with the membrane enzymes.

Heptinstall, *et. al*. (70) reported that the amount of Mg^{2+} bound by cell walls of staphylococci is significantly diminished by the presence of ester alanine. However, when staphylococci were grown in the presence of high NaCl concentration, the ester-alanine content in the walls was very much lower and the walls has a better capacity for binding Mg^{2+}. This led them to suggest that the alanyl-ester residues of the teichoic acid might function in regulating the surface charge and cation binding of the cell wall.

The difference in the nature of the binding of Mg^{2+} to the walls was explained by Archibald, *et. al.* in two different publications (45, 72) about three years later as follows. The strongly-bound Mg^{2+} ions interact with wall teichoic acid, but in the presence of alanine, the $NH3^{+}$ group of the amino acid neutralizes the charge of the adjacent phosphate, thus permitting only one of the pair of phosphate to interact with Mg^{2+}. Since a given alanyl residue may be capable of neutralizing any one of a number of phosphate groups, it was suggested that alternation of the amino group between a number of phosphates would produce changes in the position of the polymer chain at which Mg^{2+} could bind strongly to two phosphates, and this could produce a net movement of strongly bound Mg^{2+} on the polymer chain This hypothesis was further confirmed by Lambert, *et. al.* in 1975 (50). They also proposed that it is likely that membrane teichoic acids, or lipoteichoic acids, behave in a very similar way.

Despite all the complicated theories provided regarding the function of lipoteichoic acids as divalent cation scavengers, one relatively simpler theory still stands true. In whole cells lipoteichoic acid is in direct contact with the outer surface of the membrane, thus providing cations for the membrane. As a consequence of this, if the lipoteichoic acid is also in contact with wall teichoic acid, it is possible to predict and to consider the presence of an ion-exchange network from the outer surface of the wall to the cell membrane, mediated by the wall and lipoteichoic acid, as reviewed by Lambert *et. al.* (13).

In the case of *Bacillus subtilis*, Schirner, *et. al.* (23) showed that the absence of LTA leads to an abolishment of growth in the presence of Mn^{2+}, while on the growth in the presence of Mg^{2+} was not affected. This shows that the effect was not considered to be a general sensitivity to any divalent cations. In fact, they noticed that the absence of LTA actually reduced the cell's requirement for Mg^{2+}. These results provide yet another strong support for a model in which LTA is important in scavenging and sequestration of Mg^{2+} ions, which is also consistent with previous review by Neuhaus, *et. al.* (16).

The loss of LTA-dependent buffering zone in the cell wall allows divalent cations more immediate access to the cell surface. This leads to a lower requirement for Mg^{2+}, which is a cofactor in many enzymatic reactions within the cells, and increased susceptibility to toxic ions, in this case Mn^{2+}. It seems that Mn^{2+} can replace Mg^{2+} because of their similar physicochemical properties, but cannot play role as cofactors the way Mg^{2+} can. In other words, the Mn^{2+} substitute the spots for Mg^{2+} without being able to function the same way as Mg^{2+} can (23), which led them to believe that one of the most important function of lipoteichoic acid in the cell membrane is to maintain divalent cation homeostasis. Further studies by the same group also showed that disrupted divalent cation homeostasis as a result of the absence of LTA results in shape malformations in *Bacillus subtilis*, impaired septation and cell division, both in the vegetative state and sporulation stage.

2.5.4 Lipoteichoic acids as bacterial surface antigens

The terminology ‘antigen’ originally refers to a compound that has the ability to induce the formation of antibody. This definition now is limited to ‘immunogens’, where ‘antigen’ now is defined as a compound that has the ability to react with an antibody and give a demonstrable and observable effect, like precipitation, or change of color (42). Different antibodies may differ in their reactivity in different testing procedures. That being said, it is possible that response to different immunogens may be due to the difference in detection method (73).

The development of how researchers were able to determine the function of lipoteichoic acids as surface antigens went way back to the early 1970’s, where Van Driel, *et. al.* (26) discovered that *Lactobacillus fermenti* and *Lactobacillus casei* whole cells reacted differently with antibodies specific for LTA. They found that only *L. fermenti* cells were adsorbed and agglutinated by LTA-specific antiserums. This led them to use a more sensitive method to pinpoint the location of LTA and how it functions as surface antigens. The method itself involves electron microscopy, where cells were treated with antiserum and goat antibodies conjugated with ferritin and to rabbit Immunoglobulin G (IgG). *L. casei* showed some surface adsorption of teichoic acid antibody, but it was more irregular in distribution and significantly less than the labeling shown by *L. fermenti*.

This difference led them to propose a new model for the location of LTA, namely the wall-membrane model, where LTA is envisioned as embedded in the plasma membrane at their hydrophobic glycolipid ends, while the long polar glycerol phosphate chains extended into the polysaccharide and peptidoglycan network of the cell wall. In some cases, these chains may come close enough to the surface of the cell wall to act as surface antigens (26).

This result showed that lipoteichoic acids can function as a surface component. However, this condition is affected by several factors, namely the thickness of the cell wall

and the degree of peptidoglycan cross linking, the length of glycerol phosphate chain of LTA, and also the conformation of the chain within the ionic environment of the cell wall, as later shown by Wicken, *et. al.* a few years later (42).

So far it has been confirmed and reported that lipoteichoic acid has at least several numbers of potential determinants, namely glycerol phosphate backbone sequence, carbohydrates and D-alanyl substituents and the glycolipid (20, 26). This means that lipoteichoic acids carry different carbohydrate components that can be detected by antibodies specific for the backbone. Antibodies to lipiteichoic acid from Group D and Group N streptococci are generally specific for carbohydrate constituents. This is also true for antibodies from Group F lactobacilli. However *Lactobacillus casei* LTA gives antibodies that are specific for glycerol phosphate backbone sequence. That being said, the practice may not be as clear-cut as stated due to the fact that lipoteichoic acids that contain carbohydrate constituents may also demonstrate antibody response that are specific for the glycerol phosphate backbone (20, 42).

2.5.5 Lipoteichoic acids as adhesins

So far, the terminology ‘adhesions’ is used to describe a relatively stable, irreversible attachment of bacteria to surfaces. In light of this, any structures responsible for such adhesive activities is defined as an ‘adhesin’ (74). Many bacteria are enveloped with an additional matrix of polymers known as the glycocalyx (75, 76), and one of the major components in glycocalyx is lipoteichoic acid. This matrix is a highly hydrated structure and plays role, not only in cell viability, but according to a review by Neuhaus, *et. al.*, also in adherence, access of macromolecules and ions, and virulence (16). In several bacteria, coalescence of adjacent glycocalyces leads to biofilm formation (76, 77). The role of lipoteichoic acid in biofilm formation will be explained in a much greater detail in sub chapter 2.7 of this Literature Review.

On the other hand, studies regarding bacterial pathogenesis have indicated that the attachment or adherence of bacteria to mucosal surfaces is the initial event in the pathogenesis of most infectious diseases due to bacteria in animals and humans (78). In Gram negative organisms, the adhesions responsible for this mechanism resemble lectins, in a way that they recognize and bind to saccharide sequences in epithelial cell glycoconjugates (79, 80). Gram positive organisms have somewhat a different binding mechanism and binding components, and one of the most widely investigsted organisms is the streptococci.

Streptococcus pneumoniae been suggested to bind to oropharyngeal epithelial cells by recognizing a portion of the glycolipid, namely GlcNAcβ1→3Gal as the receptor (81). In a series of experiment performed by Andersson, *et. al.* (82), they discovered that *S. pneumonia* produces an adhesion which can form a link between components of the pneumococcal cell surface and carbohydrate receptor structures on the orophharyngeal epithelial cells. They also confirmed that adherence was reduced by trypsin, pepsin, heat and periodate, which suggested that the adhesion was a protein, or a molecule strongly associated with protein-like or protein-containing surface components sensitive to these treatments.

Group A streptococci causes a variety of diseases, including pharyngitis, tonsillitis, impetigo, scarlet fever, pneumonia, puerperal sepsis and myositis. They are also suspected of contributing to certain neurological disorders, such as obsessive/compulsive behavior, as reviewed by Courtney, *et. al.* (83). In group A streptococci, the functional adhesion has been proposed to consist of lipoteichoic acid complexes to M-protein, or another protein via its polyglycerol phosphate part, where it presents the fatty acid as a binding site (84, 85).

Courtney, *et. al.* (86) evaluated the roles of M protein and lipoteichoic acids in the adherence process using purified lipoteichoic acid, recombinant M protein, fragments of

M protein or synthetic peptides copying defined regions of M protein. They discovered that the adherence of group A streptococci to buccal and pharyngeal cells was not dependent in the presence of M protein on the surface of the cells. However, M protein does promote the adherence of streptococci to Hep-2 cells. Further experiments showed that lipoteichoic acid inhibited streptococcal attachment to buccal and pharyngeal cells, and blocked attachment to Hep-2 cells. Combined with their previous publication (84) and others (87, 88), they came to the conclusion that group A streptococci can utilize at least two adhesins for attachment to certain host cells. The molecular mechanisms employed may be dependent on the type of host cell used in adherence tests, and that attachment of streptococci to host cells may also be dependent on the type of streptococcal strain used in the studies.

In *Streptococcus pyogenes*, a member of group A streptococci, LTA is the first adhesion that was purified and found to block their attachment to host cells, with fibronectin identified as its receptor (84, 89). Since then, at least 17 additional surface components have been suggested to play role in adhesion of group A streptococci (83).

Similar results have also been reported in staphylocci, although somewhat contradictory to what have been reported in *Streptococcus pyogenes*. In *Staphylococcus aureus*, for example, it was shown that staphylococcal lipoteichoic acid markedly reduced adherence to buccal cells in vitro, suggesting that lipoteichoic acid mediates adherence by the bacterium. Deacylation recovers the adherence, suggesting that fatty acids on the molecule is essential to binding (90). Lipid moiety of lipoteichoic acid also has a central role in the adherence of *Staphylococcus epidermidis* to fibrin-platelet clots in vitro (91).

At first glance, different sets of published data on adhesins can be confusing and seemingly inconsistent, if not contradictory. However, scientists believe that most, if not all, of the data are correct. Hasty *et. al*. (92) proposed a theoretical framework that group A streptococci utilize multiple adhesins to adhere to host cells and that adhesion occurs

in a two-step mechanism. In the first step, streptococci must overcome the electrostatic repulsion that separates their surface from the surface of the host cells. It is proposed that LTA mediates this initial step for most, if not all, group A streptococci. After this step is completed, adhesion will be relatively weak and reversible, where it is not sufficient to provide tissue tropism. The second step is dependent upon different adhesins, like M or R protein and this has to take place in order to achieve firm or perhaps even a functionally irreversible adhesion that is tissue-specific. However, it is also important to mention that the utilization of one adhesion does not preclude the use of other adhesins (83).

It is needless to state that in this bacterial group, the expression of adhesins is controlled by a number of regulatory genes, as reviewed extensively by Courtney, *et. al.* (83). These genes will be differentially expressed depending on what environmental signals the bacteria receive and the growth phase they are on.

2.5.6 Lipoteichoic acid and cell hydrophobicity

Adherence properties of bacteria are highly dependent on the surface properties. Therefore, the surface properties of microorganisms will determine the outcome of host-parasite interaction. Studies have shown that the hydrophobic characteristics of bacteria play an important, if not central, role in their interaction with mammalian cells (93, 94).

In addition to being widely studied for their adherence properties, Group A streptococci are also known to have a strong tendency to hydrophobic interaction (95), which in the end contributes to the knowledge regarding their adherence properties. It has been shown also that the hydrophobic, or the lipid, portion of lipoteichoic acids is essential for their binding to cell membranes (87).

Originally, it was suggested that in group A streptococci, M protein is the one responsible for the hydrophobicity of the cells (95, 96). However, more recent studies showed that there is no significant difference in adherence between streptococci strains

with and without M protein (89, 97). This led Miorner, *et. al.* to believe the importance of further characterizing the surface structures of group A streptococci responsible for cell hydrophobicity (96). They found that the hydrophobic surface structure on group A streptococci either consists of a protein or molecules linked to proteins. A direct comparison between surface hydrophobicity and lipoteichoic acid was made and it showed that there is a correlation between the hydrophobic affinity partition of the bacteria in polymer two-phase systems and the quantity of surface lipotaichoic acid, indicating that it plays a major role in conferring hydrophobicity to group A streptococci. Further experiment also showed them that the hydrophobic affinity expressed by the lipoteichoic acid extract is due to the lipoteichoic acids itself rather than a protein bound to it. However, they also believed that it is not wise to rule out the possibility for other molecules to contribute to the hydrophobicity of group A streptococci.

2.5.7 Lipoteichoic acids in bacterial virulence and infection

Other Gram positive bacteria, such as *Staphylococcus aureus* can cause pulmonary inflammation. In relation to this, lipoteichoic acids, in addition to peptidoglycan, has been shown to induce inflammatory response by binding to Surfactant Protein A (SP-A) and Surfactant Protein D (SP-D), the two surfactant proteins that are believed to play important role in the innate immunity of the lung (98). Many pathogenic organisms can be bound by Sp-D and Sp-A in vitro. As a result of the binding, microorganisms can aggregate, and in many cases, this leads to enhanced killing and clearance by phagocytic cells. This group showed that lipoteichoic acid from *Bacillus subtilis* was bound by SP-D but not SP-A. In light of this, it is possible that SP-A binds to other glycoconjugates present on the cell wall. On the other hand, it cannot be fully excluded that lipoteichoic acid can be bound by SP-A. All and all, this group concluded that Gram positive bacteria are bound by SP-D via its binding to the bacterial surface components, namely LTA and

peptidoglycan. This interaction was calcium-specific and could be inhibited by carbohydrates.

Septic shock is commonly observed in bacterial infections. In general, little is known about the mechanism by which Gram positive organisms induce septic shock. One study showed that lipoteichoic acid in combination with peptidoglycan from *Staphylococcus aureus* act in synergy to cause septic shock and multiple organ failure in anesthesized rats (99). As these two components are commonly found in other Gram positive organisms, their joint activity may be able to explain the initiation of Gram positive septic shock in general.

Another leading cause of neonatal sepsis and meningitis is *Streptococcus agalactiae*, of the group B streptococci, or GBS. Lipoteichoic acid has been shown to mediate the adherence of this organism to the host cells. Mutants of *S. agalactiae* that are deficient of D-alanyl ester have a greatly decreased virulence in mouse and neonatal rat models (100, 101). In *Streptococcus mutans*, lipoteichoic acid plays role in the adherence of the bacteria to the hydroxylapatite of the tooth surface. This interaction is one of the factors that govern the formation of dental biofilms (51).

A clear correlation between the D-alanyl ester content of lipoteichoic acid and bacterial virulence has also been established in *Listeria monocytogenes*. Abachin, *et. al.* (102) were able to establish that inactivation of *dlt* operon that leads to the absence of D-alanine in lipoteichoic acid severely impaired the virulence of this organism in a mouse infection model. The mutant deficient of D-alanine showed no morphological alterations, and its growth rate was similar to that of the wild type strain. However, the adherence of the mutant strains to macrophages and human epithelial cells was strongly restricted. This result supported the hypothesis that D-alanyl-LTA is required for entry of the bacterium into the host cell (103)

Another compelling proof that lipoteichoic acid plays role in bacterial virulence was shown by Fittipaldi, *et. al.* (104). They were able to show that the absence of D-alanylation in lipoteichoic acid increases the susceptibility of *Streptococcus suis* to the action of cationic antimicrobial peptides. The mutant that is deficient in D-alanylation of its lipotaichoic acid was efficiently killed by porcine neutrophils and showed diminished adherence to porcine brain microvascular endothelial cells. Further studies also showed that the mutant deficient of lipoteichoic acid D-alanylation was attenuated in mouse infection model, showing a decreased ability to escape immune clearance mechanisms and an impaired capacity to move across host barriers. All of these suggest that D-alanylation in lipoteichoic acid is an important factor in the virulence of *Streptococcus suis*.

However, there are also indications that lipoteichoic acid may not play as significant of a role in bacterial virulence. Unlike lipopolysaccharide as an almost exclusive virulence factor in Gram negative organisms, Gram positive organisms induce tissue damage by elaborating many cytotoxic factors, and lipoteichoic acid would only be one of them. Proof of a role of lipotaichoic acid as a virulence factor at least in animal models in most cases require demonstration of its presence together with peptidoglycan during or after infections, as shown in later review (105).

2.5.8 Lipoteichoic acid and their interaction with autolytic enzymes

Autolytic enzymes are believed to play an important role during cell growth and division. They are also believed to be involved in cell wall turnover, or the secretion of cell wall fragments into the extracellular fluid by hydrolysis of bonds in the peptidoglycan (106). Wall teichoic acids have been shown to bind strongly to certain autolytic enzymes, and it has been suggested that they may, in certain cases, be involved in the localization of autolytic enzymes in the wall and in the modulation of their activity (107).

Several groups of Gram positive organisms, such as lactobacilli and streptococci have been known to spontaneously secrete lipoteichoic acid into the culture medium during exponential growth (43, 108, 109). Under conditions unfavorable for growth, such as in the presence of suitable antibiotics, autolytic activity may lead to the dissolution of the cell wall (110).

Lipoteichoic acid has also been reported as a powerful inhibitor of autolytic enzyme in *Pneumococcus* (111). Later in the future, it was shown that the absence of positively charged D-alanine ester in the lipoteichoic acid of *dlt* mutant strain seemed to reduce the rate of autolysis in *Staphylococcus aureus* due to an increase in the net negative charge (112). This is because the cationic autolysin binds to the anionic portion of lipoteichoic acid, which is regarded as a control and regulatory mechanism that reduces its activity.

It is known that lipoteichoic acids and other membrane components are able to prevent, or at least inhibit cell lysis under these unfavorable conditions (52, 53, 113). And based on these findings, it has been frequently proposed that amphiphiles such as lipoteichoic acids may also play role in the in-vivo regulation of autolytic activity and that the secretory process may be involved in the control of autolysis and cell division (53, 114).

2.5.9 Lipoteichoic acid in antibiotic resistance

In several Gram positive organisms, there is a single *dlt* operon encoding the genes responsible for the incorporation of D-alanine into teichoic acid (16, 115, 116), and that its organization is almost identical in all of them (117). Fabretti, *et. al.* (118) were able to confirm that the inactivation of genes within this operon in *Enterococcus faecalis* results in the complete absence or reduction of teichoic acid D-alanine esters.

They were also able to confirm that the absence of D-alanine results in the increased cell susceptibility toward several antibiotics, namely nisin, polymixin B and

colistin. Although they did not observe increased autolysis in *E. faecalis*, the susceptibility to cationic antimicrobial peptides (CAMPs) has been shown to increase in the *dlt* mutants of several Gram positive bacteria, such as *Staphylococcus aureus* (46), *Streptococcus agalactiae* (117), *Streptococcus pyogenes* (115), and *Listeria monocytogenes* (102).

Gutmann *et. al.* (119) somewhat were able to confirm that penicillin-tolerance and modification of lipoteichoic acid is associated with expression of vancomycin resistance in VanB-type *Enterococcus faecium* D366. In *Enterococcus faecium* of the VanB class, resistance to glycopeptides is characterized by resistance to vancomycin and susceptibility to teicoplanin. They were able to show that a derivative strain, namely MT9, did not experience neither lysis nor killing when treated with 128 µg/mL penicillin (eight times the MIC), while the same treatment performed toward D366 resulted in lysis and 2-log reduction in CFU. The comparison of the lipoteichoic acids from both strains showed no obvious difference either in the chain length or in the extent or pattern of glycosylation. However, the D-alanine ester content in strain MT9 was nearly twice as high as that in strain D366. The D-alanine esters introduce positively charged amino groups into the otherwise negatively charged teichoic acids.

In *Bacillus subtilis*, the absence of D-alanine from the polymers has been reported to cause an alteration of the activity of autolytic enzymes, which are considered to bind to teichoic acids by ionic interactions (120). This result confirmed what was proposed Gutmann, *et. al.* (119) before, that is doubling the alanine ester content observed for lipoteichoic acid when vancomycin resistance is expressed would lower the autolysin binding capacity of lipoteichoic acid, which would then affect a step in the pathway that triggers the endogenous as well as the penicillin-induced lytic process.

Other studies have also shown that the absence of D-alanine from lipoteichoic acid also significantly increases the sensitivity of *Bacillus subtilis* to antibiotics, such as methicillin (120) and lysozyme (23). This shows that lipoteichoic acid also provides

somewhat a protective layer that restricts the access of many bioactive agents to sensitive sites in the cell envelops.

Peschel, *et. al.* (112) observed somewhat a similar phenomenon in *Staphylococcus aureus*. They compared the MICs of various antibiotics for *S. aureus* SA113 wild type, the *ΔdltA* mutant strain and *ΔdltA* complemented strain. While mutant was only slightly more sensitive (less than twofold) than the wild type to methicillin, cefazolin, erythromycin and tetracycline, a considerable increase of susceptibility (more than three-fold) to the glycopeptide antibiotics vancomycin, teicoplanin and balhimycin was observed. Their studies demonstrated that teichoic acids have a considerable influence on the susceptibility to vancomycin and other glycopeptides antibiotics, which is consistent with what Gutmann, *et. al.* observed (119). They also found that the mutant strains have increased binding capacity for vancomycin, which may at least in part be responsible for the sensitivity of the mutant to glycopeptide antibiotics. The absence of D-alanine from lipoteichoic acid has also been shown to increase susceptibility to methicillin in *Bacillus subtilis* (120).

Another study by Peschel, *et. al.* (46) showed that inactivation of *dlt* operon that leads to the absence of D-alanine results in an increased sensitivity of *Staphylococcus aureus* and *Staphylococcus xylosus* mutants to defensins, protegrins, tachyplasins, and other antimicrobial peptides. The enhanced sensitivity is most likely due to the higher net polyanionic charge in the cells deficient of D-alanine, which lead to the hypothesis that D-alanylation in Gram positive organisms provides some kind of protection against cationic antimicrobial components. This is also proven by the fact that increased D-alanylation of both wall and lipoteichoic acid confers resistance to cationic antimicrobial peptides in *Staphylococcus aureus*. Since many Gram positive species possess *dlt* operon, it is also a very firm hypothesis that D-alanylation of lipoteichoic acid could be a common

mechanism for resisting peptides produced by other Gram positive bacteria, such as lactococcin, nisin, and subtilin (121).

Sometime in the mid 1980's, the first antibiotic of the lipopeptide class, namely daptomycin, was described as active against Gram positive bacteria in the presence of 50 µg/mL calcium ions (122). Canepari, *et. al.* (123, 124) were able to produce evidence in favor of the use of lipoteichoic acid synthesis as a target for the action of this particular antibiotic. Daptomycin has revealed a much higher bactericidal activity against staphylococci and enterococci than that demonstrated by vancomycin and β-lactam antibiotics (122). It is also well known and has been discussed in the previous pages of this report that lipoteichoic acid plays a role in bacterial colonization, which is a prerequisite for the ability of microorganisms to trigger infection and that adherent bacteria are less susceptible to antibiotics. Therefore they argued that antibiotics that inhibit lipoteichoic acid synthesis will act against the major determinants of bacterial virulence and prevent bacteria from proliferating in the adherent state.

Despite the finding by Canepari, *et. al.* establishing lipoteichoic acid as a target for antimicrobial action (123, 124), at least a group of researchers demonstrated a contradictory result several years later. Laganas, *et. al.* (125) showed that daptomycin inhibited all macromolecular synthesis in *Staphylococcus aureus, Enterococcus faecalis* and *Enterococcus hirae* without kinetic or dose specificity for lipoteichoic acid. Daptomycin remained bactericidal in the absence of ongoing lipoteichoic acid synthesis, which led them to believe that inhibiton of lipoteichoic acid synthesis is apparently not the mechanism of action of daptomycin in these pathogens, thus lending support to the hypothesis that daptomycin acts via the dissipation of bacterial membrane potential in these clinically relevant pathogens. Further investigation would be necessary to rule out a role for daptomycin-mediated inhibition of lipoteichoic acid biosynthesis in other pathogens.

2.5.10 Lipoteichoic acid and environmental stress

Lipoteichoic acid (LTA) is often compared to lipopolysaccharide (LPS) of Gram negative bacteria. However, previous findings makes it more appropriate to compare LTA to osmoregulated periplasmic glucans (OPGs) of Gram negative bacteria. This is because both LTA and OPGs have similarities in enzymes involved in their synthesis, their cellular location within the cells and their function in osmoprotection (126).

OPGs has been shown to accumulate in the Gram negative periplasm in low osmolarity medium, and are thought to protect bacteria under these conditions. In *Salmonella enterica* serovar Typhimurium, inability to synthesize OPGs has been shown to have no significant impact on the organism's LPS pattern, or its ability to survive stress condition, such as the presence of antimicrobial peptides, detergents, and other environmental stress related to pH and nutrient-deficiency. However, OPGs in *S.* Typhimurium contribute towards growth and motility under low osmolarity growth conditions (127).

In Gram positive cells, the presence of lipoteichoic acid is thought to affect the surface properties of the cells, along with other surface components, such as proteins and wall teichoic acid (128). In *Staphylococcus aureus,* it has been found that LTA-deficient cells can grow at 30^{o}C, but not at 37^{o}C. At the higher temperature, LTA is essential for colony formation, viability, and resistance to low-osmolarity conditions. This shows that LTA is important for osmotic regulation in *S. aureus* and other LTA-containing Gram positive organisms, as also reviewed by Percy, *et. al*. (126). LTA contains anionic polymers of phosphoglycerol, and thus releases fixed anions on the outside of the cell membrane. LTA and its counter ions could provide local osmotic pressure on the outside of the cell membrane and could eventually reduce osmotic stress. This might be why *S. aureus* lacking in LTA can be rescued in high osmolarity medium, or by an increase in the cellular c-di-AMP concentration implicated in the control of ion transport (129).

Listeria monocytogenes has been shown to have high ability to adapt to environmental stress, be it from high acidity, salt concentration and low temperature, and these are the major factors in the recent ascent to prominence of *L. monocytogenes* as a foodborne pathogen as reviewed by Bereksi, *et. al.* (130) and Ko, *et. al.* (131). There is even evidence that *L. monocytogenes* may survive even on particles as dry as dust, or particles of organic material (132). However, survival at low pH and high salt concentration was strongly temperature dependent (133), where minimum pH values that allowed survival after 4 weeks (from an initial concentration 10^4 cells) were 4.66 at 30^{o}C, 4.36 at 10^{o}C, and 4.19 at 5^{o}C. Low salt concentrations (4-6%) improved survival at limiting pH values, while higher salt concentrations reduced their survival. Earlier publications (134) showed that *L. monocytogenes* is able to resist up to 14% NaCl concentration when grown at 15^{o}C and 30^{o}C. On the other hand, it was shown later on that the lag times for *L. monocytogenes* Scott A was prolonged at higher NaCl concentrations when incubated at 22^{o}C ; 18 hours at 0% NaCl, 29 hours at 5% NaCl, and 96 hours at 10% NaCl (130). Osmotic stress also leads to a decrease in intracellular pH of *L. monocytogenes*, which eventually leads to an impaired recovery on solid medium (135). Low temperature has also been shown to influence the ability of *L. monocytogenes* to grow at low pH levels (136).

L. monocytogenes does not compete well in mixed cultures and is susceptible to bacteriolysins. Fod handling and storage practices, such as refrigeration, that eliminate or suppress competitors therefore promote the growth of *L. monocytogenes*. Since high osmolarity and low temperature are conditions that favor *L. monocytogenes* over its competitors, the processes of osmotic adaptation and low temperature adaptation are crucial to its importance as a foodborne pathogen, as reviewed by Ko, *et. al.* (131) and one mechanism of osmotic stress adaptation commonly found in bacteria involves intracellular accumulation of organic compounds called osmolytes (137, 138), which

contribute to a counterbalancing in osmotic pressure. Intracellular accumulation of osmolytes, which can occur either by transport from the growth medium, confer tolerance to high osmotic stress.

Like several other organisms, *L. monocytogenes* accumulates glycine betaine, a ubiquitous and effective osmolyte, intracellularly when grown under osmotic stress (139). However, it also accumulates glycine betaine when grown under chill stress at refrigeration temperatures. Ko, *et. al.* (131) were able to show that glycine betaine confers not only chill tolerance but also osmotic tolerance in *L. monocytogenes*, where its intracellular concentration increases almost 6-fold when cells are grown at 4^{o}C with the addition of 8% NaCl compared to without NaCl addition.

To date, three osmolyte transporters, that is Gbu, OpuC, and BetL (140) dedicated to betaine and carnitine uptake in *L. monocytogenes* have been identified and characterized, and Wemekamp-Kamphuis, *et. al.* (141) were able to confirm that each transporter is induced to different degrees upon cold shock in *L. monocytogenes* LO28. There is a fourth putative osmolyte transporter system recognized as OpuB and consists of 2 genes in *L. monocytogenes* EGD-e. However, despite being transcriptionally up-regulated upon cold shock, this system showed no significant contribution to listerial chill tolerance.

2.6 Lipoteichoic acid in *Listeria monocytogenes*

As previously stated, lipoteichoic acid (LTA) is typically obtained from Gram positive organisms, including *Listeria monocytogenes* by extraction process using phenol-water partition and gel filtration chromatography (142), or by a combination of physical cell disruption and chemical treatment (22).

Like other LTA isolated from other Gram positive organisms, the major components of LTA in *L. monocytogenes* are phosphate, glycerol, fatty acids, galactose

and glucose, where galactose is present in excess of glucose. In addition to that, as determined by Hether and Jackson (142) the average of chain length of LTA from *Listeria* spp is about 19 glycerol phosphate units. Amphiphiles such as LTA and lipopoysaccgarides (LPS) may appear to have high molecular weights as observed in gel filtration chromatography because they form micelles to exclude water from the hydrophobic regions of the polymers (14, 44).

It has been extensively explained that LTA, being an amphiphilic molecule, has both a hydrophilic and hydrophobic region. The hydrophobic region, or the lipid moiety is usually a glycolipid or a phosphoglycolipid of the type normally present in the cell membrane (44). In *Listeria* spp., based on the characterization of four different *Listeria* strains performed by Uchikawa, *et. al.* (143), the lipid portion is determined to be 3(1)-(2'-*O*-α-D-galactopyranosyl-α-D-glucopyranosyl)-1(3),2-diacylglycerol and 3(1)-[6'-phosphatidyl-2'-*O*-(α-D-galactopyranosyl)-α-D-glucopyranosyl]-1(3),2-diacylglycerol . The hydrophilic chains are 1,3-linked polyglycerol phosphate, with approximately one glycosyl side chain per lipoteichoic acid molecule. This is similar to previous findings by Hether and Jackson (142).

Lipoteichoic acid in *L. monocytogenes* is a linear polyglycerolphosphate polymer attached to the membrane by the glycolipid Gal-Glc-DAG. The free hydroxyl group can be esterified with D-alanine (D-Ala) or glycosylated with galactose (Gal) and the glucose moiety of Gal-Glc-DAG can be exchanged with lipid at position 6 with a phosphatidyl group. The most abundant fatty acids in the glycolipid and the phosphatidyl substituent are C17 and C15, as explained earlier by Hether and Jackson (142) and Uchikawa, *et. al.* (143), which was re-iterated by Webb, *et. al.* some years later (144).

Dehus, *et. al.* (145) found two distinct structural variants of LTA in *L. monocytogenes* strain ATCC 43251 using nuclear magnetic resonance and mass spectroscopy. Both LTA variants consisted of poly-glycerophosphate backbone with

different lengths. One LTA variant (referred to as LTA2) possessed a second diacyl-glycerol moiety linked to the disaccharide via a phosphodiester bond. They also found that growth temperature had a strong influence on the expression levels of both LTA variants in the cell wall. The amount of LTA2 was ten times higher for *L. monocytogenes* cultivated at 37^{o}C, compared to that cultivated at room temperature. However, the amount of LTA1 (or the variant that is commonly known in other Gram positive bacteria like *Staphylococcus aureus* or *Streptococcus pneumoniae*) is relatively comparable in both growth temperatures. They were also able to confirm that the increase amount of LTA2 at higher growth temperature does not stem from the temperature-dependent enzyme activity, but a product of specialized enzyme activity that has involved, most probably for the purpose and importance of *L. monocytogenes* virulence.

L. monocytogenes is considered facultative intracellular microorganisms, capable of invading and surviving in most host cells, including epithelial cells (146). It is probable that cell wall-associated surface proteins play an important role in the process of adherence and internalization of *L. monocytogenes*.

In other Gram positive organisms, like *Bacillus subtilis* and *Staphylococcus aureus,* the enzyme YpfP has been characterized and determined to be involved in the synthesis of glycolipids and lipid anchor for LTA (147-149). In *Enterococcus faecalis*, the enzyme BgsA has been recognized as a glycosyl transferase responsible for the addition of the second glucose moiety, which later was shown to have a significant effect on biofilm formation (150). In *Streptococcus agalactiae*, this enzyme was known to be Gbs0682, or designated as IagA and it is associated with invasion (151).

In light of this, so far, little has been done and known about LTA and glycolipid synthesis in *L. monocytogenes*. Genetic studies of LTA biosynthesis have shown the incorporation of D-alanyl residues into LTA requires the activity of four gene products, namely DltA, DltB, DltC and DltD, which are encoded by the *dlt* operon. This has been

shown in various Gram positive organisms other than *L. monocytogenes*, such as *Bacillus subtilis* (116), *Streptococcus gordonii* (152), *Staphylococcus aureus* (46) and *Lactobacillus casei* (153).

Based on this, Abachin *et. al.* (102) constructed a D-alanyl deficient LTA mutant of *L. monocytogenes* by knocking off the first gene in the operon (*dltA*). They discovered that the virulence of this mutant was severely impaired in the mouse model and that the adherence of the mutant to various cell lines was strongly restricted. This led them to believe that the D-alanylation of the LTA contributes to the virulence of this particular pathogen. The same phenomena was also shown in a later publication, where the D-alanylation of LTA contributes to the virulence of a different Gram positive organism, namely *Streptococcus suis* (104).

It has also been reported that the internalin B protein (InlB) of *L. monocytogenes* is a non-covalently attached cell surface protein, and it is required for entry into various host cells. InlB also binds to LTA and is retained at the bacterial surface in this manner (154, 155).

Since the LTA glycolipid anchor in *L. monocytogenes* consists of Gal-Glc-DAG, Webb, *et. al.* (144) hypothesize that there are two distinct glycosyl transferases required for its synthesis similar to that observed for *Enterococcus faecalis* and *Streptococcus agalactiae.* They did a BLAST search against *L. monocytogenes* EGD-e genome, using *S. agalactiae* IagA protein and *E. faecalis* BgsA as query sequences. They were able to identify Lmo2554 and Lmo2554 proteins as the closest homologues. This led them to suggest that both Lmo2555 and Lmo2554 could encode glycosyltransferases specific for UDP-glucose and UDP-galactose and responsible for Glc-DAG and Gal-Glc-DAG synthesis, respectively. They renamed these proteins LafA and LafB for LTA anchor formation proteins A and B.

In *Staphylococcus aureus*, the enzyme responsible for the synthesis of polyglycerolphosphate backbone has been recognized as LtaS (59), and two proteins with a high degree of similarity to this enzyme was also identified by Webb, *et. al.* (144) in *L. monocytogenes*, namely Lmo0927 and Lmo0644. Inactivation of both genes led to a complete absence of LTA, which led them to hypothesize that Lmo0927 is responsible for the synthesis of the polyglycerolphosphate backbone chain. Further experiment showed that Lmo0644 functions as LTA primase, which initiates LTA synthesis by transferring the first glycerolphosphate subunit into the glycolipid. After that, a second enzyme, namely LTA synthase, would extend the chain to form the polyglycerolphosphate backbone. They were able to confirm Lmo0927 to function as such, thus proposing the name LtaP or LTA primase for Lmo0927 and LtaS or LTA synthase for Lmo0644.

Further down the road, they were able to show that LafA (Lmo2555) and LafB (Lmo2554) are necessary for the production of Glc-DAG and Gal-Glc-DAG, respectively. These enzymes likely use the nucleotide-activated sugars UDP-glucose and UDP-galactose as substrates. They observed a profound reduction in the overall amount of LTA in the absence of Gal-Glc-DAG. The simplest explanation that they suggested for the reduction in LTA production is that because the enzymes that are needed for the formation of the polyglycerolphosphate polymer cannot efficiently initiate LTA synthesis in the absence of glycolipids. In *L. monocytogenes*, it seems clear that in the absence of the glycolipids, neither LtaP nor LtaS can efficiently initiate polyglycerolphosphate backbone synthesis. Therefore, it is very likely that LtaP and LtaS could either have a specific recognition site for glycolipids, or alternatively there is a special constraint that could dictate which lipid can be used for LTA synthesis.

Webb, *et. al.* (144) were able to provide evidence that *L. monocytogenes* uses two different enzyme systems for the synthesis of LTA and based on the fact that they still observed LTA being synthesized, although at a reduced level when both *ltaP* and *lafA*

were deleted. This is very different from *S. aureus*, where it only requires one single enzyme system in its LTA synthesis (156). It is still unclear, however, why some bacteria use only one single system while the others requires multiple enzyme systems.

2.7 Lipoteichoic acids and biofilm formation

As previously stated, surface properties of the cells are important in altering these adherence properties, which eventually determines the outcome of a host-parasite interaction (96). Previous studies have also shown that the hydrophobic character of bacteria plays a central role in their interaction with mammalian cells (93, 94).

One of the key components of biofilm formation is the ability of cells to adhere to a solid surface, and lipoteichoic acid is presumed to be important in biofilm formation in its ability to alter the adherence properties of the organisms. Some of the publications that discuss the importance of LTA in biofilm formation of several Gram positive organisms can be found below.

2.7.1 Enterococci

Enterococci are considered to be commensals in human and animal microflora. In recent years, this group have emerged as one of the leading cause of nosocomial infections, with *Enterococcus faecalis* being the major clinical isolates in most cases (157). In addition to that, biofilm formation is found to be critical for the pathogenesis of many of these infections.

Lipoteichoic acid polymer of most Gram positive bacteria is nonstoichiometrically replaced with D-alanine at the C-2 glycerol position, and some previous publications have recognized the presence of a single *dlt* operon encoding the genes responsible for D-alanine incorporation (115, 116). Based on this, Fabretti, *et. al.* (118) used targeted mutagenesis to inactivate the first enzyme involved in the contribution of teichoic acid

alanylation to biofilm formation on polystyrene surface. They were able to confirm that D-alanylation of teichoic acid is involved in biofilm formation and suggested that this is due to the lack of D-alanine esters on the teichoic acid, which causes a stronger negative net charge on the bacterial cell surface, thus affecting several bacterial properties. This is because D-alanine esters introduce positively charged groups into the negatively charged teichoic acids, thus the absence of the D-alanine esters increases the negative net charge of the cells.

They also compared the difference in cell morphology between the wild type and the deletion mutant by transmission electron microscopy. Interestingly, they found no obvious morphological difference between wild type and mutant strain; they both exhibited extracellular polysaccharide material and a normal cell wall.

In addition to the *dlt* operon, different mechanisms have been recognized and explained for biofilm formation by *E. faecalis*. One of them is the involvement of membrane glycolipids and LTA content. Theilacker, *et. al.* (158) were able to confirm the presence of *E. faecalis* gene EF2891 and its similarity (48% sequence identity) to Al*dgs*, a gene that encodes a diglucosyl-diacylglycerol (DGlcDAG) synthetase in *Acheloplasma laidlawii* and several other Gram positive bacteria. This gene also shares high similarity with *iagA*, a gene required for anchoring LTA to the cell membrane and for invasion across the blood-brain barrier by *Streptococcus agalactiae*. Because of its association with biofilm formation, Theilacker, *et. al*. designated this gene as *b*iofilm-associated *g*lycolipid synthesis A (*bgsA*). Using BLAST analysis, they were also able to identify a second putative glucosyltransferase, immediately downstream of *bgsA*.

In order to characterize the role of *bgs*A, they created a non polar deletion mutant, where they delete 863 bp of the gene. This eventually led to an almost complete loss in the ability to form biofilm on plastic surfaces. Through series of experiment, they were able to characterize the function of BgsA, which is a glycolyltransferase synthesizing DGlcDAG,

a glycolipid and LTA precursor involved in biofilm accumulation. When the gene was inactivated, DGlcDAG synthesis was completely abolished, and this glycolipid was replaced by α-monoglucosyl-diacylglycerol (MGlcDAG). This indicates that BgsA is a (1→2) glucosyl transferase synthesizing the glucosylation of MGlcDAG into DGlcDAG. As the latter is major part of glycolipid in *E. faecalis*, it is somewhat expected that there would be a change in the concentration of other polar lipids of the cell membrane. However, analysis of total phospholipids and amonophospholipids by thin layer chromatography did not reveal any major differences (158).

Following these results, and knowing that the gene *bgsB* which is located directly downstream of *bgsA* has never been characterized, the same group pursued its role in glycolipid metabolism in LTA biosynthesis and bacterial physiology (150). They started by creating another deletion mutant using a strategy similar to what they had done previously to *bgsA* (158), deleting 790 bp from the targeted *bgsB* gene. They were able to confirm that the *bgsB* encodes for a glycosyltransferase that glycosylates DAG to form MGlcDAG. And as shown previously (158), MGlcDAG is the substrate of BgsA, which adds a second glucose to form DGlcDAG. Since BgsA does not accept DAG as a substrate, consequently, inactivation of BgsB results in the loss of all glycolipids from the cell membrane.

Similar to deletion of *bgsA*, deletion of *bgsB* also led to impaired biofilm formation. They also were able to show that the defect in biofilm formation was not a result of a decreased initial attachment, but it was due to defective accumulation of biofilm mass after initial attachment (150). Over a period of 24 hours, biofilm mass of wild-type bacteria on polystyrene grew in a linear fashion. In contrast, the amount of biofilm produced by *bgsA* and *bgsB* mutants remained constant at the level of initial attachment.

Combining all these results together, it does not seem too far-fetched to conclude that reduction in biofilm formation does not necessarily come from alteration in cell

morphology in *E. faecalis*. Since the deletion of the first gene in the *dlt* operon leads to the reduction in biofilm formation and D-alanylation of the LTA, it can be concluded that there is a correlation between the two. The deletion of *bgsA* gene did affect LTA synthesis, where the *E. faecalis* mutant produces longer polyglycerolphosphate portion of LTA. Since this eventually led to a severe reduction of biofilm formation in the mutants, it is a clear indication that LTA somewhat plays role in the formation of biofilm in *E. faecalis*.

2.7.2 Staphylococci

Teichoic acid structure and biological activities have been studied to some extent in *Staphylococcus aureus* as wall teichoic acids (WTA) and lipoteichoic acids (LTA) seem to contribute to the virulence potential of this foodborne pathogen (159, 160). Lipoteichoic acid has also been confirmed to be an essential molecule for the viability of the cell, namely when it comes to growth and division (59).

In staphylococci, the structure of lipoteichoic acid is recognized as polyglycerolphosphate attached to a glycolipid anchor, namely β-D-Glc*p*-(1→6)-β-D-Glc*p*'-(1→3)-diacylglycerol or DGlcDAG (22), and YpfP protein is a glycolipid synthase which mediates the synthesis of DGlcDAG in this bacteria (147).

Recently Grundling, *et. al.* (59) were able to confirm the presence of *ltaA* gene, located directly downstream of *ypfP.* Later they were also able to confirm that *ltaA* gene is involved in LTA biosynthesis by translocating DGlcDAG from the inner to the outer leaflet of the cytoplasmic membrane.

Following these results, with the knowledge that *ypfP* gene has been shown to be essential for DGlcDAG biosynthesis (149), Fedtke, *et. al.* generated a deletion mutant of *ypfP* gene from *S. aureus* SA113 in order to better understand LTA biosynthesis and function (156). They found that *ypfP* deletion mutant still produces lipoteichoic acid, although the LTA content was 87% reduced, and with the polymer attached to

diacylglycerol (DAG) instead of DGlcDAG. However, the 87% reduction in LTA content has no major impact on in vitro cell growth, indicating that the remaining 13% of LTA is sufficient for the mutant strain to fulfill most of the cellular functions where LTA is involved.

Based on biofilm assay performed on two different surfaces, they observed a severely reduced biofilm formation, where, contrary to the wild type strain, the mutant strain was not able to adhere to polystyrene surface. Cell hydrophobicity was also significantly increased in the mutant strain. However, both wild type and mutant strain were able to form biofilm on glass, which indicated that changes in hydrophobicity of either bacteria or biomaterials are crucial for the ability of the cells to form biofilm.

Through series of experiment performed with *S. aureus* SA113, Fedtke, *et. al*. were able to conclude that LTA content governs the physicochemical surface properties of *S. aureus* and enables biofilm formation (156). However, despite all of this, it is important to note that the different consequences of *ypfP* gene deletion same gene deletion using different strain of *S. aureus* may yield different result when it comes to LTA content, cell hydrophobicity and biofilm formation. *ypfP* deletion mutant from *S. aureus* RN4220 yielded increased amount of LTA, although with changes in chemical composition (149). This mutant strain also exhibited the same cell hydrophobicity with the wild type (156). Thus it is important to remember the different consequences of *ypfP* deletion were clearly dependent on the strain background. It might be safe to speculate that subtle, yet unknown differences in the LTA polymerization process determine the impact of the lack of glycolipid on LTA biosynthesis rates.

2.7.3 Bacilli

The multiple structural changes of the cell envelopes in Gram positive bacteria make it difficult to delineate the specific function of glycolipids and LTA in Enterococci

group. On top of that, the importance of LTA in cell growth makes it more difficult to create mutants that are devoid of this component. However, in other Gram positive organisms, like *Bacillus subtilis*, mutations of genes involved in the precursors of the glycolipid and LTA synthesis also affect biofilm formation (161, 162).

In *Bacillus subtilis*, the gene called *gtaA* encodes the enzyme for the transfer of glucosyl group from UDP-glucose (UDP-Glc) to the polyglycerolphosphate portion of the major wall teichoic acid (163, 164). The precursor of UDP-Glc is α-glucose-1-phosphate (α-Glc 1P), which is isomerized from glucose 6-phosphate (Glc 6-P) by α-phosphoglucomutase, or α-PGM. Mutations of another gene, namely *gtaC* led to α-PGM deficiency (165).

Lazarevic, *et. al.* (162) constructed a mutant strain of *Bacillus subtilis*, where *gtaC* is mutated, in order to determine the importance of α-PGM in cell morphology and biofilm formation. They discovered that biofilm formation by the mutated strains was greatly diminished compared to the wild type strain. This reduction in biofilm formation was apparently not due to poor growth of the mutants, since the OD_{600} measured after 46 and 70-hour incubation, as well as viable cell counts after 70 hours of incubation were comparable to the values obtained from the wild type. Based on this, they suggested that the biofilm formation deficiency may be due to the absence of UDP-Glc, which may be the precursor of a putative compound required for biofilm formation. Contrary to previous knowledge, they found that the incorporation of the polyglycerolphosphate portion of LTA was comparable to that of the wild type.

Like what has been observed in other Gram positive organisms, the relationship between lipoteichoic acid and biofilm formation in *Bacillus subtilis* is somewhat indirect. Up to now, the relationship can be explained as follows. Mutation of *gtaC* gene led to a deficiency in α-PGM, which eventually led to reduction of biofilm formation. The deficiency in α-PGM also causes a deficiency in α-Glc 1P, which is a precursor of UDP-Glc. UDP-

Glc itself is known as a glucosyl donor for the synthesis of phosphate-containing anionic envelope polymers of Gram positive organisms. This glucosyl group is transferred from UDP-Glc to the polyglycerolphosphate section of the major wall teichoic acid. Knowing all of these, and connecting this with the function of lipoteichoic acid in wall teichoic acid synthesis, it is feasible to hypothesize that lipoteichoic acid plays role in biofilm formation of *Bacillus subtilis*.

2.8 Literature review conclusion and project rationale

So far, it has been extensively discussed and confirmed that lipoteichoic acids are present in most Gram positive organisms and their role is of major importance in the functionality of the cells. One of them is their role as adhesins, which later affects the virulence and pathogenicity of certain human and foodborne pathogens, including *L. monocytogenes* (102). This role is of major concerns to food industries in particular, because cell adherence determines the ability of cells to form bacterial biofilms, which increases the resistance of cells to cleaners, sanitizers and antimicrobials.

There are a number of publications highlighting the importance of LTA in biofilm formation of some Gram positive organisms, namely enterococci, staphylococci and bacilli, although the relationship is somewhat indirect. However, little is known about this relationship in *L. monocytogenes*. So far, two genes, namely *lmo2555* and *lmo2554* have been identified to be responsible for the synthesis of the two glycolipid backbones Glc-DAG and Gal-Glc-DAG by acting as glycosyltransferases required for the formation of these two major components.

Our lab has observed that there is a severely-reduced biofilm formation when transposon mutagenesis is performed along this operon, which leads us to believe that either one or more of these three genes plays a critical role in biofilm formation (166). However, it is yet to be determined how these two genes influence biofilm formation. In

addition to that, it is also very likely that the operon where these two genes are located also contains another gene, namely *lmo2553*, which is located directly downstream of *lmo2554.* This is the basis for us to argue the importance of creating an in-frame deletion mutant of each of these genes in order to get better information on the role of lipoteichoic acid in *L. monocytogenes* biofilm formation.

CHAPTER III

OBJECTIVES

1. Creating in-frame deletion mutants of 3 genes, that is *lmo2555, lmo2554* and *lmo2553*.
2. Constructing *Δlmo2555, Δlmo2554* and *Δlmo2553* complemented strains.
3. Quantifying lipoteichoic acid in both wild type, mutant and complemented strains in order to see how the deletion of each gene affect the synthesis of lipoteichoic acid.
4. Evaluating the influence of *lmo2555, lmo2554* and *lmo2553* upon biofilm formation under static and flow conditions and on a variety of surfaces.
5. Visualizing cells within *L. monocytogenes* biofilm community, both wild type, mutant and complemented strains using confocal laser scanning microscopy.
6. Evaluating the importance of LTA in growth of *L. monocytogenes* at low temperature.
7. Evaluating the importance of LTA on the survival of *L. monocytogenes* exposed to low temperature and high osmotic stress.

CHAPTER IV

MATERIALS AND METHODS

4.1 Bacterial Strains and Growth Conditions

All frozen stock cultures were mixed with glycerol (final concentration 25%) and kept at -80^{o}C. For working cultures, a one percent aliquot was transferred into Tryptic Soy Broth (TSB, BD Bacto, Sparks, MD) supplemented with 0.6% yeast extract (YE, BD Bacto, Sparks, MD)(TSBYE) and incubated overnight at 32^{o}C. On the following day, one loop of overnight culture was streaked on Tryptic Soy Agar (TSA, BD Bacto, Sparks, MD) supplemented with 0.6% Yeast Extract. Plates were incubated overnight at 32^{o}C. These plates were referred to as working stock and was kept at 4^{o}C for up to one month. One day prior to the actual experiment, one colony was taken from working stock and transferred into 10mL TSBYE and incubated overnight at 32^{o}C. The overnight grown culture was later used for experiments. When needed, appropriate antibiotics were added to media at the concentrations specified in Table 4.1.

All *Escherichia coli* strains used in this project were grown overnight in Luria-Bertani (LB) broth (BD Difco, Sparks, MD) with shaking, with the following with the appropriate antibiotic as described in Table 4.1. *Escherichia coli* strain DH5α electro competent cells for the purpose of electroporation was obtained from New England Biolabs (Ipswich, MA) and kept frozen at -80^{o}C. All bacterial strains used and generated in this project are recorded in Table 4.1.

Table 4.1. Bacterial strains used and generated in this study

Designation	Description, phenotype and growth conditions[1]	Genotype	Source
	Escherichia coli		
DH5α	Obtained from New England Biolabs (Ipswitch, MA), for the purpose of creating recombinant plasmids	N/A	
S17-1	Conjugation donor, for the purpose of complementation	N/A	(167)
IMC755	*E. coli* DH5α, with pKSV7 plasmid carrying deletion fragment of *lmo2555*, Amp^{100}	pKSV7::Δlmo2555	This study
IMC754	*E. coli* DH5α, with pKSV7 plasmid carrying deletion fragment of *lmo2554*, Amp^{100}	pKSV7:: Δlmo2554	This study
IMC753	*E. coli* DH5α, with pKSV7 plasmid carrying deletion fragment of *lmo2553*, Amp^{100}	pKSV7:: Δlmo2553	This study
IMC17	*E. coli* S17-1, with pIMK2 plasmid, Kan^{50}	pIMK2	This study

Table 4.1. Bacterial strains used and generated in this study (continued)

Designation	Description, phenotype and growth conditions[1]	Genotype	Source
	Escherichia coli (continued)		
IMC255	*E. coli* S17-1, with pIMK2 plasmid carrying *lmo2555* gene, Kan[50]	pIMK2::*lmo2555*	This study
IMC254	*E. coli* S17-1, with pIMK2 plasmid carrying *lmo2554* gene, Kan[50]	pIMK2::*lmo2554*	This study
IMC253	*E. coli* S17-1, with pIMK2 plasmid carrying *lmo2553* gene, Kan[50]	pIMK2::lmo2553	This study
	Listeria monocytogenes		
LM21	FSL-J1-225, ScottA, serotype 4b	Wild type	(168)
IM55	LM21 with In-frame deletion mutant of *lmo2555*, reduced biofilm formation	*Δlmo2555*	This study
IM54	LM21 with in-frame deletion mutant of *lmo2554*, reduced biofilm formation	*Δlmo2554*	This study

Table 4.1. Bacterial strains used and generated in this study (continued)

Designation	Description, phenotype and growth conditions[1]	Genotype	Source
	Listeria monocytogenes (continued)		
IM53	LM21 with in-frame deletion mutant of *lmo2553*, reduced biofilm formation	*Δlmo2553*	This study
IM21B	LM21 strain carrying pIMK2 vector, biofilm formation comparable to wild type strain, Kan[50]	LM21,pIMK2::tRNA^{Arg}	This study
IM255	LM21 strain carrying pTIR2555 recombinant plasmid, biofilm formation comparable to wild type strain, Kan[50]	LM21, pTIR255::tRNA^{Arg}	This study
IM254	LM21 strain carrying pTIR2554 recombinant plasmid, biofilm formation comparable to wild type strain, Kan[50]	LM21, pTIR254::tRNA^{Arg}	This study
IM253	LM21 strain carrying pTIR2553 recombinant plasmid, biofilm formation comparable to wild type strain, Kan[50]	LM21,pTIR253::tRNA^{Arg}	This study
IM55B	IM55 strain carrying pIMK2 plasmid, reduced biofilm formation, Kan[50]	IM55, pIMK2::tRNA^{Arg}	This study

Table 4.1. Bacterial strains used and generated in this study (continued)

Designation	Description, phenotype and growth conditions[1]	Genotype	Source
	Listeria monocytogenes (continued)		
IM55C	Unsuccessful complementation of IM55 mutant; IM55 strain carrying pTIR2555 plasmid, Kan^{50}	IM55, pTIR255::$tRNA^{Arg}$	This study
IM54B	IM54 strain carrying pIMK2 plasmid, reduced biofilm formation, Kan^{50}	IM54, pIMK2::$tRNA^{Arg}$	This study
IM54C	Complementation of IM54 mutant; IM54 strain carrying pTIR2554 plasmid, Kan^{50}	IM54, pTIR254::$tRNA^{Arg}$	This study
IM53B	IM53 strain carrying pIMK2 plasmid, reduced biofilm formation, Kan^{50}	IM53, pIMK2::$tRNA^{Arg}$	This study
IM53C	Unsuccessful complementation of IM53 mutant; IM53 strain carrying pTIR2553 plasmid, Kan^{50}	IM53, pTIR253::$tRNA^{Arg}$	This study

[1]Kan^{50}: supplementation of media with 50µg/mL kanamycin

Amp^{100}: supplementation of media with 100µg/mL ampicillin

4.2 Primer sequences, PCR reactions and gel electrophoresis

Primer sequences used in this project, both for construction of all in-frame deletion mutants and complementation purposes (restriction enzymes underlined) are shown in Table 4.2. Primers were obtained from Sigma-Aldrich (St. Louis, MO).

TaKaRa Ex-Taq (Clontech Laboratories, Mountain View, CA) and was used as DNA polymerase in most PCR reactions that involved downstream reactions. Maxime iTaq PCR Mix Kit (Intron Biotechnology, New York, NY) was used for all screening and confirmation purposes. All PCR reactions are performed using a C1000 Thermal Cycler (BioRad, Hercules, CA).

Gel electrophoresis was performed using 0.8% SeaKem LE Agarose Gel (Lonza, Allendale, NJ). DNA ladders (2-kb, Sigma-Aldrich, St. Louis, MO), and/ or a 1-kb ladder (New England Biolabs, Ipswich, MA) were used as gel electrophoresis size standards. Ethidium bromide (Fisher Scientific, Pittsburgh, PA) was added to the gel to a final concentration of 2ng/mL for the purpose of visualization under UV light.

4.3 Plasmid and DNA Extraction

Plasmid extractions were performed using either Wizard *Plus* Minipreps DNA Purification System (Promega, Madison, WI) during the construction of in-frame deletion mutants, or Zyppy Plasmid Extraction Kit (Zymo Research, Irvine, CA) in the construction of complementation strains. DNA extractions from Agarose Gel after electrophoresis were performed using Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). After each reaction, restriction endonucleases and buffers are removed using DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA).

Table 4.2. Oligonucleotides used in this study

Primer (description)[a]	Oligonucleotide sequence (5'–3')[b]	Restriction site
In-frame deletion		
CIL-51(*lmo2555* up FWD)	ATAT*TCTAGA*TTCAAGAGTTTGGTTCTAACGG	XbaI
CIL-52(*lmo2555* up REV)	ATAT*GGATCC*AAACGGAATACTTGGTAAACGA	BamHI
CIL-53(*lmo2555* down FWD)	ATAT*GGATCC*AATGGCCGAGTAAAAGTGGAAT	BamHI
CIL-54(*lmo2555* down REV)	ATAT*GAATTC*ATAGAAAACTACGCGGGCAATC	EcoRI
CIL-41(*lmo2554* up FWD)	ATAT*TCTAGA*CAATGAAGATGATGAGCTTGCA	XbaI
CIL-42(*lmo2554* up REV)	ATAT*GGATCC*GAAATCAACGGTATGGTAATGC	BamHI
CIL-43(*lmo2554* down FWD)	ATAT*GGATCC*GTAAAAGAAGTAGATAACCCTG	BamHI
CIL-44(*lmo2554* down REV)	ATAT*GAATTC*ATAGAAAACTACGCGGGCAATC	EcoRI
CIL-31(*lmo2553* up FWD)	ATAT*TCTAGA*TTATTTCCAATGGCGATTTTG	XbaI
CIL-32 (*lmo2553* up REV)	ATAT*GGATCC*AATGAAGCCGCTGCTAATGG	BamHI
CIL-33 (*lmo2553* down FWD)	ATAT*GGATCC*GGTCCGGCAAAACTCCTAAT	BamHI
CIL-34(*lmo2553* down REV)	ATAT*GAATTC*AAAGTGTGTGAACATCCGAAA	EcoRI

Table 4.2 (continued). Oligonucleotides used in this study

Primer (description)[a]	Oligonucleotide sequence (5'–3')[b]	Restriction site
Complementation		
IML-51(*lmo2555* upstream)	ATAT*CCATGG*GATGAATGAACCAACGAGGAA	NcoI
IML-52(*lmo2555* downstream)	ATAT*CCCGGG*GCCACACCCTGACCTTTTAC	XmaI
IML-41 (*lmo2554* upstream)	ATAT*CCATGG*CACGTTCAAAGGAAAGAGAGG	NcoI
IML-42 (*lmo2554* downstream)	ATAT*CCCGGG*AGCCGATGCTAATGGCTAAT	XmaI
IML-31(*lmo2553* upstream)	ATAT*CCATGG*AGAGGATCGACTGGCTGAAA	NcoI
IML-32 (*lmo2553* downstream)	ATAT*CCCGGG*TCCCTAAAAAGCTGGGAACA	XmaI

[a)]FWD, forward; REV, reverse

[b)]Underline fonts indicate restriction sites

Table 4.3. Plasmids used and generated in this study

Plasmid	Description and use	Reference
pKSV7	Temperature-sensitive, integration shuttle vector in *E. coli/Listeria*, , 6.9kb, Amp^r	(169)
pTIR755	Shuttle vector pKSV7 carrying *lmo2555* deletion fragment at XbaI and EcoRI restriction sites	This study
pTIR754	Shuttle vector pKSV7 carrying *lmo2554* deletion fragment at XbaI and EcoRI restriction sites	This study
pTIR753	Shuttle vector pKSV7 carrying *lmo2553* deletion fragment at XbaI and EcoRI restriction sites	This study
pIMK2	Site- specific listerial integrative vector, constitutive overexpression, 6.2kb, Kan^r	(170)
pTIR255	Integration plasmid pIMK2 carrying *lmo2555* gene at NcoI and XmaI restriction sites	This study
pTIR254	Integration plasmid pIMK2 carrying *lmo2554* gene at NcoI and XmaI restriction sites	This study
pTIR253	Integration plasmid pIMK2 carrying *lmo2553* gene at NcoI and XmaI restriction sites	This study

4.4 Creation of deleted fragments

Deletion fragments of determinants *lmo2555*, *lmo2554* and *lmo2553* were created by PCR amplification. Fragments were amplified from the upstream region and the downstream region of each target gene. The fragments were joined using a unique BamHI site introduced on the PCR primers, to create an in-frame deletion of each targeted gene with T4-DNA Ligase (Fisher Scientific, Pittsburgh, PA) at 15^{o}C for 16 hours, creating a deleted version of each gene.

4.5 Construction of recombinant plasmids

Each deleted gene fragment is double-restricted with EcoRI and XbaI, then cloned into a temperature-sensitive *E. coli/Listeria* shuttle vector pKSV7 (152), with Insert/Vector molar ratio of 1/3, using T4-DNA Ligase at 15^{o}C for 16 hours.

4.6 Electroporation to *E. coli* DH5α electro competent cells

Approximately 100ng of recombinant plasmid was mixed with 50µL of electro competent cells and put into a 0.1-cm gap electroporation cuvette (BTX Harvard Apparatus, Holliston, MA). The mixture was electroporated using a Gene-Pulser apparatus (BioRad, Hercules, CA) with the following parameters: 1.7kV, 200Ω resistance, and 25µF capacitance for 4-6 seconds, generating a time constant between 4 to 4.5 miliseconds. A volume of 450µL SOC media (171) was added to the mixture immediately after electroporation. The mixture was transferred into a sterile test tube and incubated at 37^{o}C with shaking for approximately 2 hours. The solution was then plated on LB agar supplemented with 100µg/mL Ampicillin, 40µg/mL X-gal (Fisher Scientific, Pittsburgh, PA) and 0.01mM IPTG (Fisher Scientific, Pittsburgh, PA) to enable blue/white selection. Plates were incubated at 37^{o}C for 48 hours. Blue colonies signified the presence of plasmid, while white colonies signified the presence of insert within the plasmid.

Insertion of deletion fragment into the plasmid was confirmed by restricting the recombinant plasmid with both restriction enzymes (XbaI and EcoRI) and running the output on a 0.8% Agarose gel. The targeted colonies with the right insert should have 2 bands, one band at 7kb that signifies the size of the plasmid, and another band at the size of the insert. Once insertion was confirmed, plasmids were transferred to LM21 via electroporation.

4.7 Electroporation into *Listeria monocytogenes* strain LM21

L. monocytogenes strain LM21 was grown overnight in 500ml TSBYE at 32^{o}C until OD_{600} reached somewhere between 0.4 and 0.6 (exponential phase). Cell pellets were obtained by centrifugation at 8,000rpm for 10 minutes at 4^{o}C. Cells were washed in ice-cold sterile double distilled water, followed by washing twice with 10% cold sterile glycerol. Pellets were then suspended with 1mL of 10% cold sterile glycerol to give a highly concentrated electrocompetent cells. Cells are divided into aliquots of 120 µl and immediately stored at -80^{o}C.

Recombinant plasmids were transformed into *L. monocytogenes* wild type strain (LM21) by means of electroporation using a BioRad Gene Pulser apparatus as described previously (section 4.6). After electroporation and 2-hour incubation in SOC media (171), suspension was plated to TSAYE supplemented with 10µg/mL chloramphenicol (Sigma-Aldrich, St. Louis, MO). Plates were then incubated at 32^{o}C for 48 hours.

4.8 Creation of in-frame deletion mutants

Transformed colonies obtained after 48-hour incubation (further referred to as transformants) were individually-transferred into 5mL TSBYE and incubated overnight at 32^{o}C. Allelic exchange was performed in a two-step process. Initially, transformants were maintained under antibiotic selection (10µg/mL chloramphenicol) at an elevated

temperature (41°C), at which the pKSV7 *ori* is no longer functional. Selection for cells with integrated plasmids by homologous recombination took place afterwards by transferring an aliquot of 10µL of cell suspension into 10mL fresh TSBYE with antibiotic selection and incubated overnight at 41ºC. This action was repeated 3 times, which allows selection of cells which had undergone homologous recombination. After 3 transfers, one loop of each suspension is streaked on a TSAYE plate supplemented with antibiotics.

Selection for colonies that had undergone allelic exchange was performed by incubating isolates without antibiotic selection. This was done as follows. Several colonies that had undergone homologous recombination were transferred into TSBYE with antibiotic selection and incubated overnight at plasmid replication permissive temperature (32ºC). An aliquot of 10µL is transferred into 10mL of fresh TSBYE and incubated for approximately 10 to 12 hours at 32ºC. After 3 transfers, the solution was diluted, plated and incubated at 32ºC. After excision of the plasmid from the chromosome, the remaining chromosomal allele should be either wild type or containing the in-frame deletion mutant.

Approximately 360 colonies were picked and tested for antibiotic sensitivity, which signifies the absence of plasmid in the chromosome. Antibiotic-sensitive colonies were tested and screened for in-frame deletion mutants by PCR amplification and gel electrophoresis. Mutants should generate a smaller size band compared to the wild type strain due to the gene deletion.

A schematic diagram of construction of deletion fragments and in frame deletion mutants with homologous recombination and allelic exchange can be found on Figure 4.1.

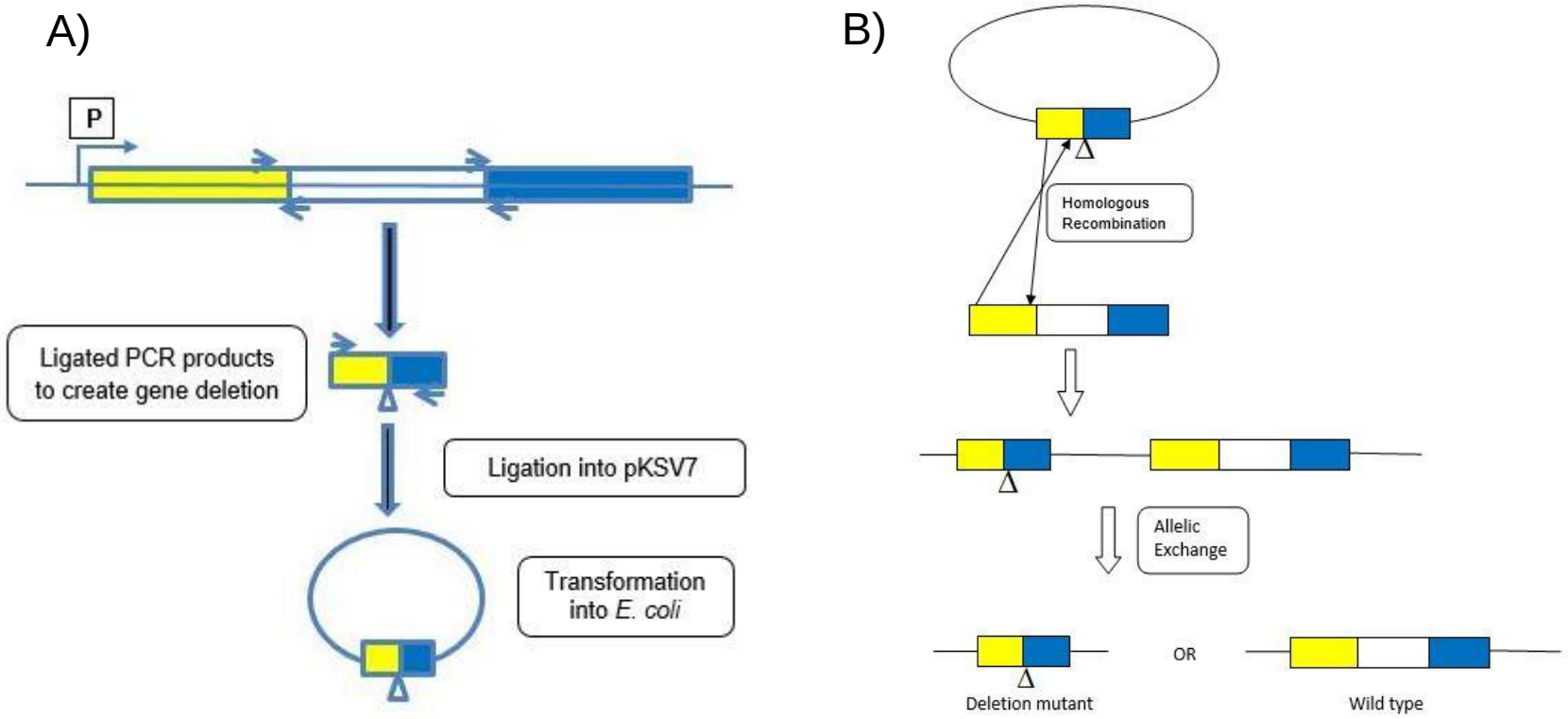


Figure 4.1. Amplification and cloning of deletion fragments (A), and generation of in-frame deletion mutants in *L. monocytogenes* via allelic exchange (B).

4.9 Complementation of in-frame deletion mutants

4.9.1 Creation of gene fragments and recombinant plasmids

Separate gene fragments, *lmo2555*, *lmo2554* and *lmo2553* were constructed by PCR amplification using IML upstream and downstream primer pairs for each gene, and LM21 strain as a template. Each primer pairs have XmaI and NcoI restriction sites to enable cloning into an integrative plasmid with multiple cloning sites (MCS). Gene fragments were cloned into a Kanamycin-resistant pIMK2 plasmid which allows for constitutive overexpression of genes from the synthetic Phelp promoter (170, 172). Vectors and recombinant plasmids were extracted using a Zyppy Plasmid Extraction Kit (Zymo Research, Irvine, CA) and confirmed by means of both PCR amplification and double digestion with NcoI and XmaI (New England Biolabs, Ipswich, MA).

Plasmids were extracted and then electroporated into *E. coli* S17-1 for the purpose of conjugation following methods that have described previously in this project (sub chapter 4.6). Plasmids used and generated in this project was recorded in Table 4.3.

4.9.2 Construction of complemented strains

Conjugation between *L. monocytogenes* and *E. coli* was performed with the following combinations: (i) blank vector into wild type and all mutant strains, and (ii) vector with insert into wild type strain and its respective deletion mutant strain. Conjugation was performed according to the method described previously (167, 173) with several modifications as explained below.

All *L. monocytogenes* (recipients) and all *E. coli* S17-1 with vectors and inserts (donors) were grown overnight with selection in their respective conditions at 32^{o}C. A volume (2.5mL) of donor suspension was mixed with 1.5mL of recipient suspension. The mixture was filtered onto a sterile 25-mm diameter mixed cellulose filter membrane with

0.45µm pore size (EMD Millipore, Billerica, MA). The membrane was aseptically removed from the filtration system, washed gently with 10mL TSBYE and transferred onto TSAYE without selection, followed by 2-hour incubation at 32°C. Each membrane was then gently resuspended into 2mL TSBYE and cells were scraped very gently using a sterile 1mL tips. An aliquot of 100s then gently resuspended intoBYE supplemented with 50µg/mL kanamycin and 20µg/mL nalidixic acid for the purpose of overnight selection and to prevent the growth of the donor *E. coli* cells, followed by incubation at 32°C. After solution showed turbidity, which signifies the presence of conjugants, an aliquot of 75µL was plated onto selective TSAYE (supplemented with 50µg/mL kanamycin and 20µg/mL nalidixic acid) and incubated overnight before streaking onto selective TSAYE in order to isolate purified transconjugates.

4.10 Lipoteichoic acid expression

A dot blot immunoassay was used for a semi-quantitative assessment of the expression of LTA on the cell surfaces. The presence of lipoteichoic acid was determined using a mouse monoclonal antibody specific for LTA from all serotypes of *L. monocytogenes* (LSBio, Seattle, WA). Strains LM21, IM21B, IM54, IM54B, and IM54C were grown overnight at 32°C. *E. coli* S17-1 was used as a negative control, grown overnight in LB broth at 37°C with shaking at 200rpm. Cell pellets were obtained by 10 minute centrifugation at 13,000rpm. Cells were concentrated ten times, re-suspended in PBS pH 7.4, and heat-killed (174). Cells were serially diluted (1:1, 1:4, 1:16, 1:64, 1:256, and 1:1024) and an aliquot of 20µL added to a well of a Bio-Dot microfiltration apparatus (Bio-Rad, Hercules, CA) and transferred by water vacuum filtration to a pre-wetted nitrocellulose membrane. Blocking was performed using a 3% (w/v) store-bought non-fat dry milk in PBST (Phosphate Buffer Saline pH7.4 with 1% Tween20). The anti-LTA antibody was diluted 1:100 in PBST with 1% bovine serum albumin (PBST-BSA).

Detection of the bound antibody was performed using an Opti-4CN Goat-anti-Mouse antibody (Bio-Rad, Hercules, CA), with the Goat-anti-Mouse-HRP conjugated secondary antibody, diluted 1/5000 in TBST-BSA. Colorimetric detection was performed using an Opti-4CN substrate and diluent that comes with the Opti-4CN detection kit (Bio-Rad, Hercules, CA).

4.11 Microtiter plate biofilm assay

Biofilm assay under static condition was performed using a PVC 96-well plate according to a method developed by Djordjevic, *et. al.* (175) and later on modified by Chang, *et. al.* (166). One colony of strains (LM21 and mutants) were grown overnight in TSBYE at 32^{o}C. One percent of the inoculum was transferred and mixed into Modified Welshimer's Broth (MWB) (176). An aliquot of 150µL was transferred into 8 wells of a round-bottom PVC 96-well plate (Corning, Tewksbury, MA) and incubated at 32^{o}C. After 48 hours, the media was removed, wells were washed with sterile distilled water five times to remove unattached cells and then air dried for approximately 15 minutes. Wells were stained with 175µL Crystal Violet (0.1%) for 45 minutes in the biological safety hood. After this, crystal violet was removed, wells were washed with sterile distilled water, and then de-stained with 200µL ethanol for 1 hour. An aliquot of 150µL was transferred into a new plate and Absorbance was measured using a microtiter plate reader (BioTek, Winooski, VT) at 570nm.

4.12 Bacterial Adherence to Hydrophobicity (BATH)

Cell hydrophobicity was evaluated according to the bacterial adhesion to hydrocarbon test described in Prachaiyo and McLandsborough (177) and performed as follows. Pellets of overnight cells grown in TSBYE were collected by centrifugation at 10,000rpm for 5 minutes. The supernatants were decanted; cell pellets were washed three

times with phosphate buffer saline (PBS), pH 7.4 prior to final suspension. For each strain 4mL of washed cells were used and a volume of 1mL of hydrocarbon (xylene or hexadecane) was added to each tube. Tubes were equilibrated for 10 minutes at 37^{o}C, vortexed full speed for 15 seconds and incubated for 30 minutes at 37^{o}C. Both incubations were carried out using a waterbath. The hydrocarbon (top) layer was removed from each tube and the absorbance of the aqueous layer was measured at 540nm using BioSpec-mini UV-visible Spectrophotometer (Shimadzu, Kyoto, Japan). Bacterial adhesion to hydrocarbon value was obtained by calculating the ratio of sample absorbance after hydrocarbon treatment to the initial absorbance prior to hydrocarbon treatment. Experiment was repeated 3 times and the absorbance is measured in duplicates.

4.13 Zeta Potential Measurement

Each strain is grown overnight in TSBYE at 32^{o}C. Cell pellets were obtained by centrifugation at 13,000rpm for 2 minutes. Pellets are washed with sterile double distilled water twice before suspended in phosphate buffer saline (137 mM Sodium chloride, 2.7 mM Potassium chloride, 10 mM Disodium hydrogen phosphate and 8 mM Potassium dihydrogen phosphate) adjusted to pH 5.8, pH 7.0 or pH 8.0 using NaOH or HCl. Zeta potential measurement was performed using a Zetamaster instrument (Malvern Instrument Ltd., Westborough, MA).

4.14 Initial Attachment Assay

One colony of each strain is grown overnight in 10mL of TSBYE at 32^{o}C. The following day, 1% overnight grown suspension was inoculated into 10mL of MWB and incubated for another 24 hours at 32^{o}C. On the day of the experiment, cell pellets were obtained by centrifugation at 13,000rpm for 5 minutes. Pellets were re-suspended in 5mL of fresh MWB and the OD_{600} was adjusted to approximately 1.0. A volume of 125µL is

inoculated into 8 wells of 3 PVC 96-well plates. Plates were incubated at 32^{o}C for 30 minutes, 2 hours and 5 hours. At each time point, one plate was taken out and initial attachment assay was performed in ways described previously (sub chapter 4.10).

4.15 Biofilm Assay under flow condition

Biofilms were grown under flow conditions using a drip flow biofilm reactor (DFR, BioSurface Technologies, Bozeman, MT). The DFR consists of a rectangular base made of polysulfone helad at a certain angle, typically 10^{o}C with the help of four adjustable legs. The unit has 4 channels, each channel holds one stainless steel slide (6.25cm^{2} surface area). Each channel has a mini-nert valve on one end, where needle is placed to deliver the media (influent), and an opening at the end of the channel where media passes through into a waste container (effluent). In DFR, the flow of media is the only acting shear force on the biofilm and a peristaltic pump is present in the assembly between the DFR unit and the media container to provide a steady flow of sterile media at a certain rate.

Biofilms were grown using DFR following the recommendation given by BioSurface Technologies, which involves two stages; batch phase for 24 hours, followed by continuous phase for another 48 hours. Prior to experiment, the DFR unit is assembled with one stainless steel chip in each channel and sterilized for 20 minutes in liquid cycle. One colony of *L. monocytogenes* (IM21B, IM254, IM54B and IM54C) were grown overnight in TSBYE at 32^{o}C. On the day of the experiment, 1mL of the overnight growth was put into 15mL of MWB as a starting inoculum for initial 24-hour batch phase. The mixture was then put into the channel. The reactor was then incubated at 32^{o}C for 24 hours. Continuous phase immediately followed, signified by the continuous flow of sterile MWB (0.8 mL/minute) into each channel for another 48 hours. Both media (TSBYE for overnight growth and MWB for biofilm growth) were supplemented with 50µg/mL Kanamycin (Sigma-Aldrich, St. Louis, MO)

Levels of cell accumulation under flow was determined using plate counts. Each stainless steel slide was aseptically removed using a sterile forceps, and was gently washed using 5mL 0.1% Buffered Peptone Water (BD Bacto, Sparks, MD). After washing, each side of the stainless steel slide was scraped for 30 seconds into a sterile 10mL 0.1% Buffered Peptone Water using a sterile teflon policeman cell scraper. The solution was then serially diluted and plated onto TSBYE using an automated plater Autoplate 4000 (Spiral Biotech, Norwood, MA). Plates were incubated overnight at 32^{o}C and colonies were counted the next day using an automatic colony counter Scan500 (Interscience, Saint Nom, France). Cell numbers were expressed in cfu/cm^2 and the experiment performed in 3 independent replications. The numbers were averaged and statistical analysis were performed in order to determine the differences among the cell numbers.

4.16 Visualization of biofilm using confocal microscope

Biofims were grown using a drip flow reactor (DFR) with 24 hour batch phase followed by 48 hour continuous phase according to the method described in 4.14. For the purpose of confocal imaging, glass microscope cover slips were used instead of stainless steel slides. Biofilms were stained using FilmTracer FM 1-43 green biofilm cell stain (Molecular Probes, Eugene, OR) following procedures provided by the company. Stain stock solution was prepared by dissolving the content of the vial with 100µL DMSO. Staining solution was prepared by diluting 10µL of stock solution with 990µL DMSO, followed by diluting 100µL into 0.9mL filter-sterilized water to reach final concentration of staining solution at 1µg/mL.

Biofilms were stained by adding the stain solution on to the sample. Samples were incubated in the dark for 30 minutes. Prior to imaging, samples were rinsed gently with filter-sterilized water. Visualization was performed using a Nikon Eclipse 80i confocal

microscope system under green channel (419/515 nm excitation/emission wavelength) with 60x oil immersion lens.

4.17 The influence of lipoteichoic acid (LTA) upon sensitivity of *L. monocytogenes* to antimicrobial compounds

The sensitivity testing to antimicrobials was tested against four antimicrobials. Two were Quarternary Ammonium Compounds (QAC) (i) benzethenium chloride (BZT) and (ii) cetylpyridinium chloride (CPC). Sensitivity to two highly charged antimicrobial surfactants were tested: lauric arginate (LAE), which is a cationic antimicrobial surfactant, and sodium dodecyl sulfate (SDS), which is an anionic antimicrobial surfactant. Both BZT and CPC were purchased from Sigma Aldrich (St. Louis, MO). Stock solutions were made by diluting BZT and CPC separately with double distilled water (ddH_2O) to 1,000ppm concentration. Stock solutions were filter-sterilized prior to use. Lauric Arginate was provided by Vedeqsa Group LAMIRSA (Terrassa, Spain) under the commercial name Mirenat-N in 10.5% concentration in propylene glycol. The solution was diluted using ddH_2O to 2,500ppm, filter-sterilized and kept in room temperature as a working stock. The working stock was then diluted into the final concentration needed in the experiment. Sodium Dodecyl Sulfate (SDS) was purchased from Sigma Aldrich (St. Louis, MO). Working stock of SDS was made by diluting SDS with ddH_2O to 10,000 ppm concentration. Working stock was then filter-sterilized and kept at room temperature until ready to use. On the day of experiment, working stock was diluted into the final concentration needed in the experiment.

The experiment was performed using a method previously described by Fox, *et. al.* (178) with several modifications. One colony of each strain (IM21B, IM254, IM54B, and IM54C) was grown overnight in TSBYE at 32^{o}C. On the following day, cell pellets were obtained by centrifugation at 13,000 rpm for 2 minutes, washed with sterile dH_2O, and re-

suspended in fresh TSBYE Kan^{50} to an OD_{600} of 0.3, and was used as the inoculum (1%) into fresh TSBYE Kan^{50} containing various concentrations of antimicrobial compounds. An aliquot of the mixture was transferred into a sterile 96-well polystyrene plate (200µL each well) and incubated at 32^{o}C. Absorbance of the suspension was measured using a microtiter plate reader at 630nm after incubation for 72 hours. MIC (Minimum Inhibitory Concentration) of each strain was defined as a concentration of QAC where turbidity was not observed, which indicated the lowest concentration of antimicrobial that inhibits each strain.

4.18 The influence of lipoteichoic acid (LTA) on the survival of *L. monocytogenes* exposed to osmotic stress at different temperatures

The effect of LTA on the survival of *L. monocytogenes* under different osmotic stress and temperature was performed by growing cells on agar adjusted to different water activities (a_w), 0.98, 0.95, 0.93, and 0.90 under different temperatures. The water activity of tempered TSAYE was adjusted using NaCl, sucrose and glycerol as solutes and measured using AquaLab water activity meter (Decagon Devices, Pullman, WA). Measured a_w with percent NaCl, sucrose and glycerol is recorded on Table 4.4. These numbers were plotted to a standard curve which was used to calculate the amount of each solute to add to TSAYE to reach a_w 0.98, 0.95, 0.93, and 0.90. The standard curve showed an almost linear relationship where the calculated R-square was 0.93 or higher for all solutes (results not shown). Standard TSAYE and reduced a_w plates were prepared with Kan^{50}. Plates were allowed to dry overnight at room temperature prior to inoculation.

One colony of each strain (IM21B, IM54B, and IM54C) were grown overnight in TSBYE at 32^{o}C. On the day of the experiment, cell pellets were obtained by centrifugation at 13,000 rpm for 2 minutes, cells were washed with sterile dH_2O, and pellets were suspended in fresh TSBYE to an OD_{600} of 0.3. A series of serial dilutions (10^{-1} – 10^{-6}) were

prepared in TSBYE. A drop (10µL) of each dilution was added into each plate, creating a row of dilutions. The inoculum cultures were plated to determine the actual CFU/10 µl in each drop Plates were then incubated at 4°C, 20°C, 32°C, and 37°C. Observation was performed daily, after 5 days for plates incubated at 32°C and 37°C, 7 days for plates incubated at 20°C, and 14 days for plates incubated at 4°C.

Cell morphology observation was also performed by growing cells in TSBYE at 4°C, 20°C, 32°C, and 37°C. Visualization of cells were performed using a Nikon Eclipse Microscope with 60x oil lens with DIC filter.

Table 4.4. Percent humectants added to TSAYE and measured water activity (a_w)

NaCl		Sucrose		Glycerol	
Percent	Measured a_w	Percent	Measured a_w	Percent	Measured a_w
0	0.998	0	0.998	0	0.998
4	0.980	5	0.998	5	0.986
5	0.977	10	0.991	6	0.978
6	0.967	15	0.990	8	0.971
7	0.955	20	0.987	10	0.966
8	0.946	25	0.972	12	0.960
9	0.931	30	0.966	14	0.951
10	0.929	35	0.954	15	0.947
11	0.922	40	0.948	16	0.939
12	0.909	45	0.934	20	0.927
13	0.900	50	0.917	25	0.907
14	0.892				
15	0.883				
20	0.798				

CHAPTER V

RESULTS

Previously in our laboratory, several mutants generated by transposon mutagenesis along the *lmo2555-lmo2554-lmo2553* operon and showed a significantly reduced ability to form biofilms (166). Three of these mutants, SS33-4F, SS2-4D and SS1-4H had transposons located within the *lmo2554* gene, within the *lmo2555* gene, and in between *lmo2555* and *lmo2556*, respectively. By performing a growth curve analysis, we were able to confirm that there is no difference in growth rate between the three mutants and wild type strain, which lead us to believe that the reduced biofilm formation is not due to a lower growth rate of the mutants (Figure 5.1). From here, we proceeded to creating an in frame deletion mutant for each of the gene along the operon, to further characterize how this operon contributes to biofilm formation in *L. monocytogenes.*

5.1 Creation of in-frame deletion mutants

In-frame deletions of each individual gene (*lmo2555, lmo2554* and *lmo2553*) were created through amplification upstream and downstream of the targeted chromosomal genes using PCR (Figure 4.1). *L. monocytogenes* wild type strain (LM21) was used as a template and primer pairs along with the nucleotide sequences and restriction enzymes are tabulated in Table 4.2. The upstream and downsteram PCR fragments were ligated using a BamHI site encoded on the primers to create a deletion fragment. These fragments were ligated on to a temperature-sensitive shuttle vector pKSV7 in *E. coli*, creating *E. coli* recombinant plasmids pTIR755, pTIR754, and pTIR753 (Table 4.1, Figure 4.1).

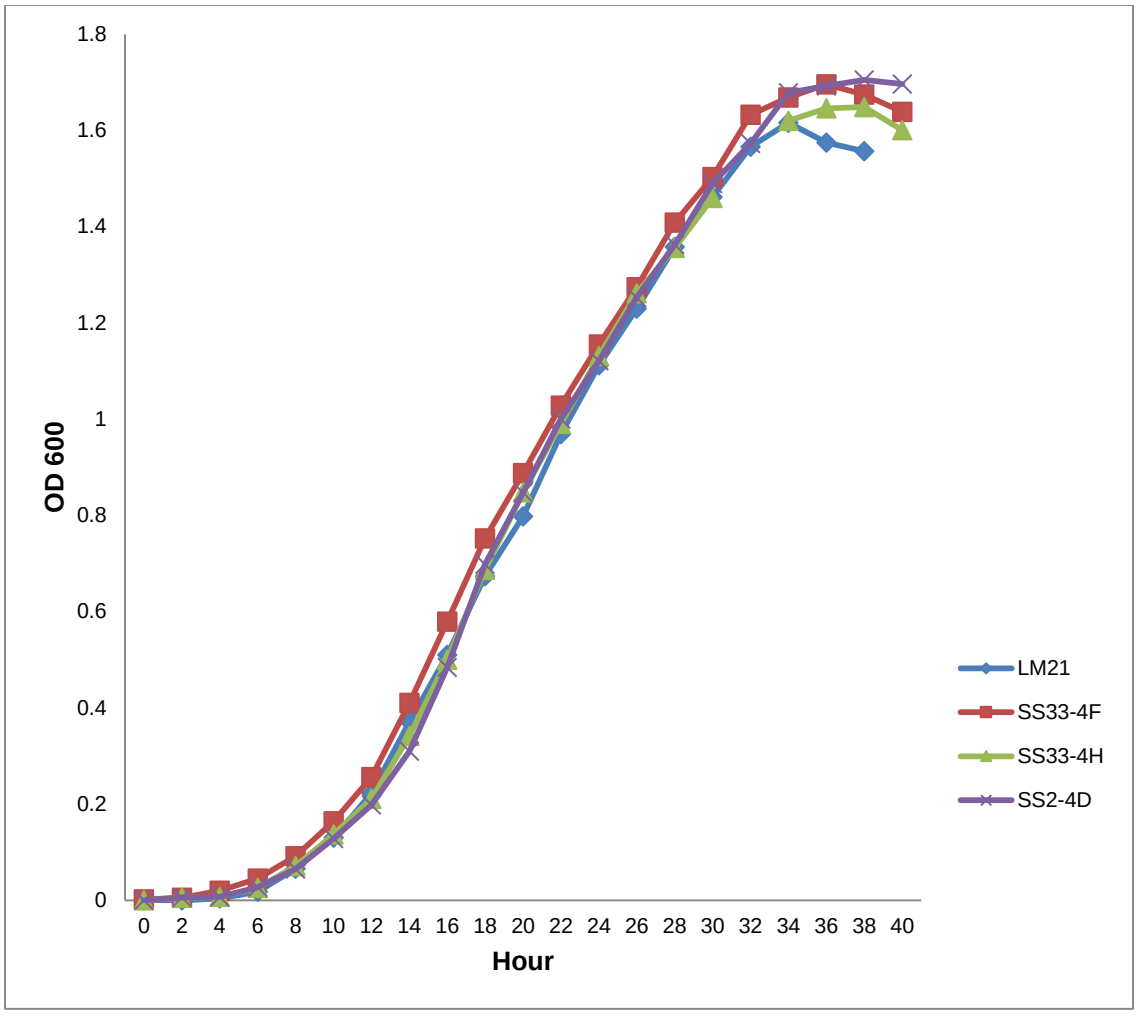


Figure 5.1. Growth curve of *L. monocytogenes* wild type strain (LM21) and 3 strains generated by transposon mutagenesis (SS33-4F, SS33-4H, and SS2-4D). One colony was incubated overnight at 32°C in TSBYE. The following day, OD was adjusted by allowing the culture to grow in fresh media until mid-exponential phase. One percent inoculum was then transferred into fresh TSBYE. OD measurement was performed every 2 hours at 600nm wavelength.

Recombinant plasmids were then transformed into *L. monocytogenes* LM21 by means of electroporation. Through series of homologous recombination and allelic exchange, approximately 360 colonies for each gene deletion were screened for antibiotic sensitivity, indicating plasmid loss. Approximately 10 colonies out of 360 were antibiotic-sensitive, and these colonies were tested to confirm gene deletion by PCR amplification and gel electrophoresis.

We were able to create individual in-frame deletion mutants of each gene in the *lmo2555-lmo2554-lmo2553* operon, by removing 840 bp, 681 bp and 831 bp from *lmo2555, lmo2554* and *lmo2553,* respectively. Consequently, in-frame deletion mutants (IM55, IM54 and IM53) showed smaller band size compared to wild type strain (LM21) due to the deletion process (Figure 5.2).

5.2 Biofilm assay under static conditions

The ability of the in-frame deletion mutant of *lmo2555, lmo2554,* and *lmo2553* (strains IM55, IM54 and IM55, respectively) to form biofilms in the PVC microtiter plate assay (Figure 5.3). Biofilm assay showed reduction in biofilm formation by approximately 67% when *lmo2555* and *lmo2554* were deleted separately and approximately 50% when *lmo2553* was deleted. Statistical analysis was performed using SAS 9.3 software. Analysis of Variance (ANOVA) showed that the difference among strains was significant at 95% confidence level. Further analysis using Tukey's HSD showed that the difference between wild type strain (LM21) and each of the mutants was highly significant at $P < 0.05$, and that there is no significant difference observed among the three deletion mutants.

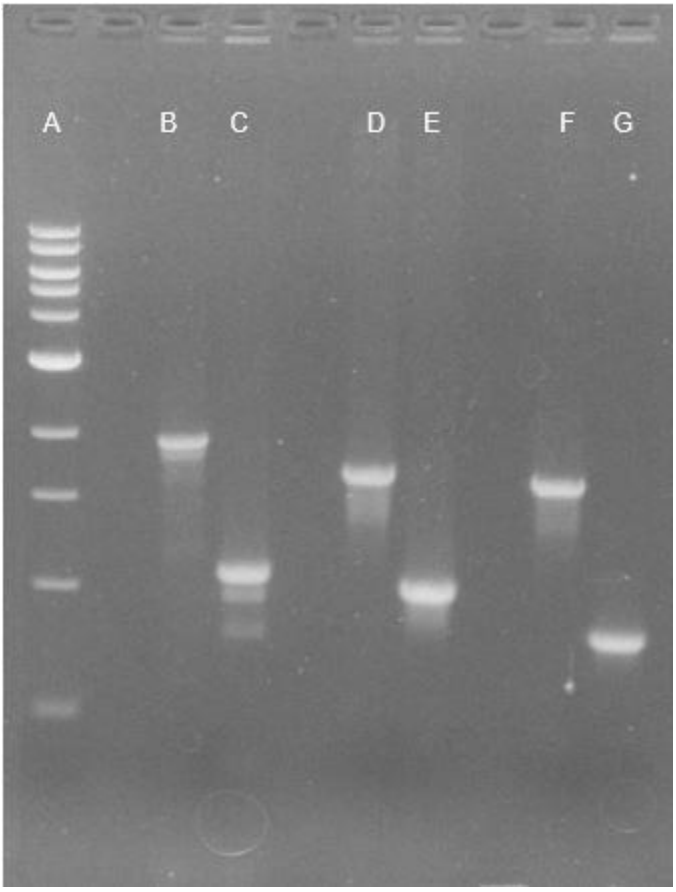
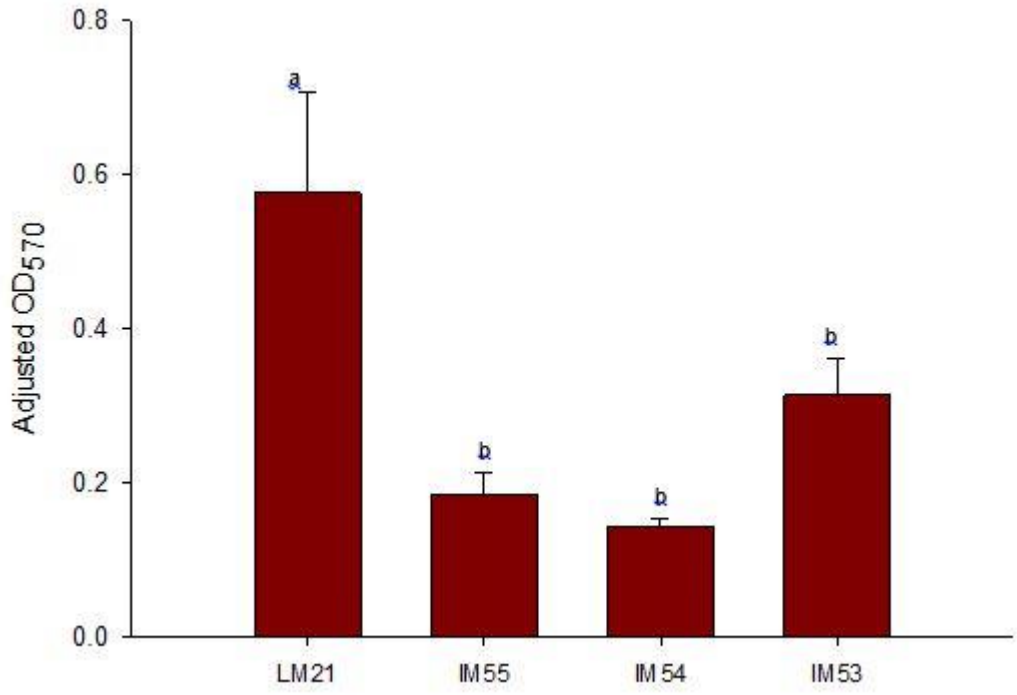


Figure 5.2. Gel electrophoresis of *L. monocytogenes* wild type and the three mutant strains. PCR reactions were performed using respective primer pairs on Table 4.1 for each mutant strains, with overnight growth of each strain used as a template. Gel electrophoresis was performed on 0.8% Agarose Gel mixed with 2ng/mL ethidium bromide.

A = DNA Ladder (10, 8, 6, 5, 4, 3, 2, 1.5, 1, 0.5kb)

B, D, F = wild type (1,883bp, 1,661bp and 1,623bp)

C, E, G = IM55 (1,043bp), IM54 (980bp), IM53 (792bp)



(Note: Characters indicated significant differences tested with Tukey's HSD at 95% confidence level)

Figure 5.3. Biofilm formation of wildtype and deletion mutants. Assay was performed on a 96-well PVC plate, according to a method developed by Djordjevic, *et. al*. (175). Cells were grown overnight in TSBYE at 32^{o}C and mixed with Modified Welshimer's Broth (176) at 0.1% concentration. Plates were incubated at 32^{o}C for approximately 48 hours. Wells were washed with sterile distilled water, stained with 0.1% crystal violet for 45 minutes and de-stained with ethanol for 1 hour. Absorbance was measured using a microtiter plate reader at 570nm.

A growth curve experiment was performed to determine if the observed reduced biofilm formation in the three deletion mutants was due to a reduction in growth rate. Growth rate was monitored by measuring optical density at 600nm in planktonic cells grown in both TSBYE and MWB (the media that was used for the biofilm assay) as shown in Figure 5.4A and 5.4B. The three deletion mutants (IM55, IM54, and IM53) showed comparable growth rate, thus we believe that the observed reduction in biofilm formation was not due to the difference in planktonic growth rate among the strains.

5.3 Initial attachment assay

An initial attachment assay was performed to determine if the reduction of biofilm was associated with a reduction in the attachment of individual cells with the PVC. The attachment assay was performed by inoculating high number of overnight growth cells onto sterile PVC well plate for 0.5, 2 or 5 hours. The initial attachment assay was performed using *L. monocytogenes* wild type strain (LM21) and the three mutant strains (IM55, IM54, and IM53). Experiment was performed in three repetitions and the OD measurement was averaged and plotted onto a graph shown on Figure 5.5. Statistical analysis showed that the difference among the strains and time points are significant. Further analysis showed that in each time point, the difference among the 4 strains was significant. Tukey's HSD analysis at 95% confidence level showed the strain IM53 did not show significant difference in OD measurement compared to the wild type (LM21).

The strains IM55 and IM54 also showed similar trends in all three time points, which showed significantly lower adhesion compared to the wild type (LM21) but not against each other. However, at 30-minute time point, OD measurement for IM55 was significantly higher than IM54. This gave us the idea that the absence of LTA may have contributed to the initial attachment of cells that preceded biofilm formation.

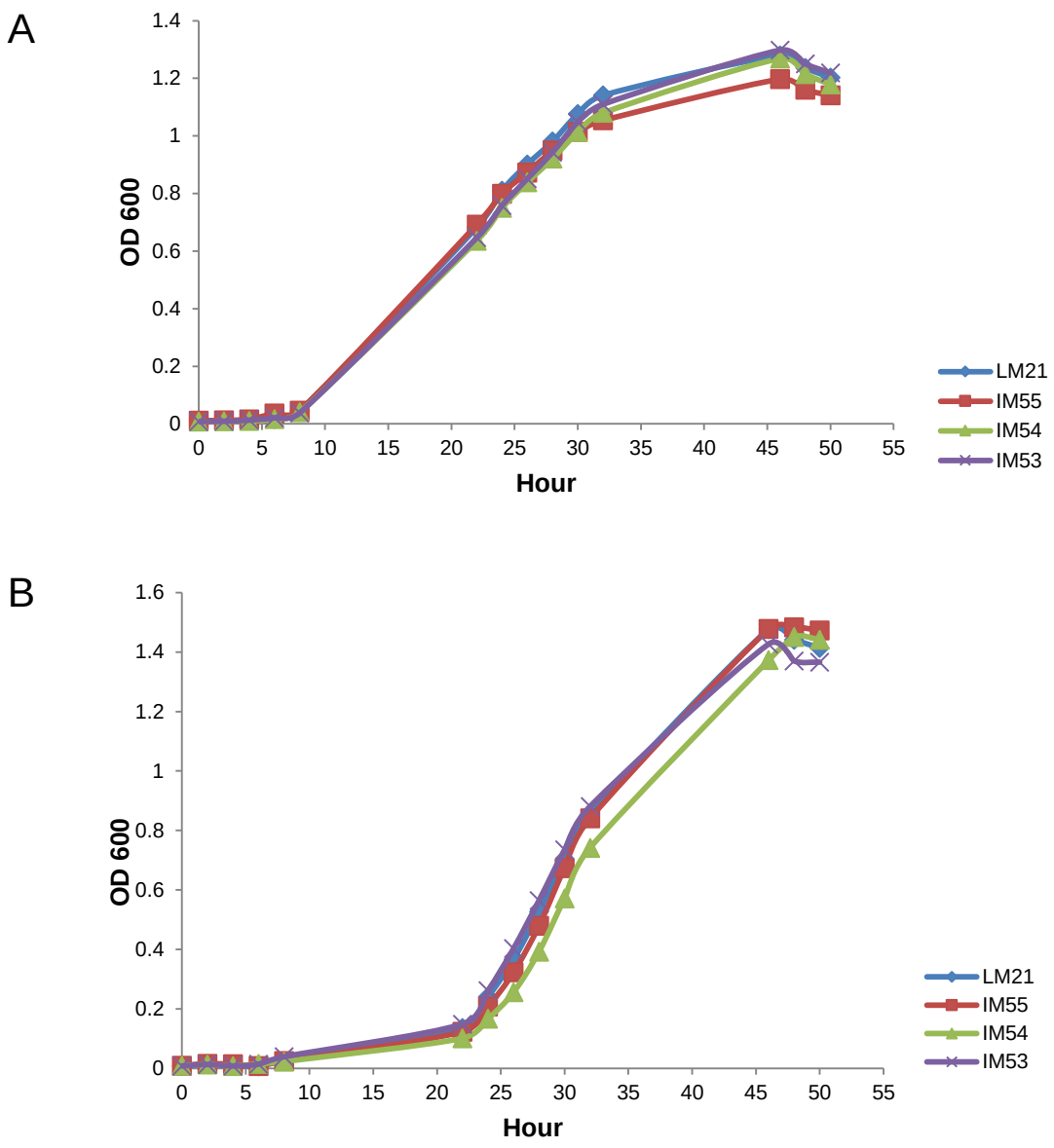
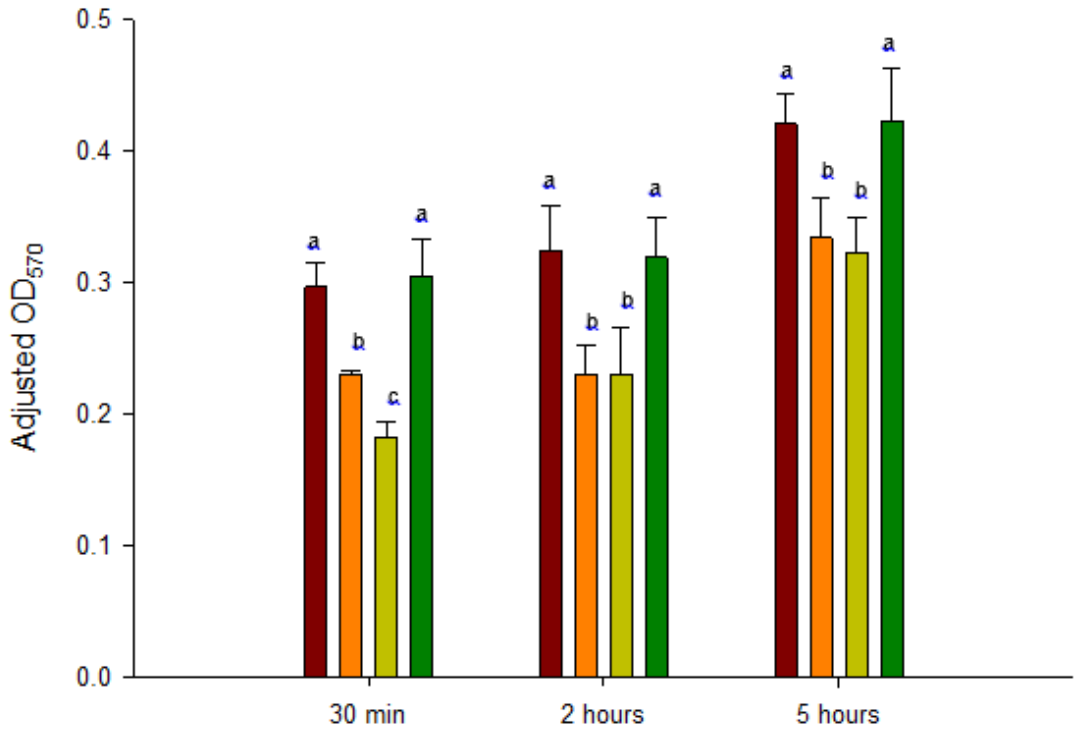


Figure 5.4. Growth curve comparison of *L. monocytogenes* wild type strain (LM21) and the three deletion strains generated in this project (IM55, IM54, and IM53). One colony was incubated overnight at 32°C in TSBYE. The following day, OD was adjusted by allowing the culture to grow in fresh media until mid-exponential phase. One percent inoculum of the exponential phase was then transferred into either fresh TSBYE (A) or MWB (B) and OD measurement was performed at 600nm wavelength.



(Note: Characters indicated significant differences tested with Tukey's HSD at 95% confidence level)

Figure 5.5 Initial Attachment Assay. *L. monocytogenes* wild type strain (LM21) and three mutant strains (IM55, IM54 and IM53) was performed following a method described by Chang, *et. al.* (145). An aliquot of 1% overnight growth was transferred into Modified Welshimer's Broth (156) and incubated for another 24 hours at 32°C. OD_{600} for all strains were adjusted to approximately 1.0, followed by incubating an aliquot of 125µL into three separate PVC 96-well plates. Plates were incubated at 32°C for 30 minutes, 2 hours and 5 hours. Wells were washed with sterile distilled water, stained with 0.1% crystal violet for 45 minutes and de-stained with ethanol for 1 hour. Absorbance was measured using a microtiter plate reader at 570nm.

5.4 Bacterial Adherence to Hydrocarbon (BATH) assay

The hydrophobicity of bacterial cells can influence initial adhesion to surfaces. Hydrophobicity of strains was assessed using the Bacterial Adherence to Hydrocarbon (BATH) assay with xylene and hexadecane as hydrocarbons, following methods previously described by Prachaiyo and McLandsborough (154). Experiment was performed in three repetitions, and for each repetition, two absorbance measurements were obtained and averaged. The results is shown in Table 5.1. Statistical analysis was performed using SAS 9.3 software. Results show that the effect of strain and hydrocarbon were significant and there is an interaction between strain and hydrocarbon. Because we are primarily interested in the difference among strains within each hydrocarbon, we separate the data based on hydrocarbon, where we established that the difference among strains in hexadecane was non-significant, and the difference among strains in xylene was significant. Mean separation using Tukey's HSD further showed the difference among strains in xylene, where in general, the adherence of wild type strain to hydrocarbon is not different from the adherence of the other three mutant strains.

5.5 Zeta potential measurement

Bacterial surface charge can influence the adhesion of cells to surfaces. The Zeta potential measurement was performed on overnight growth planktonic cells grown in TSBYE. Cells were washed with sterile Phosphate Buffer Solution (PBS) and re-suspended in PBS of different pH (pH 5.8, pH 7.0 and pH 8.0). The experiment was performed in three repetitions and for each repetition, measurement was performed in duplicates. The numbers are then averaged and compiled in the Table 5.2**.**

Table 5.1. Bacterial Adherence to Hydrocarbon (BATH) assay using xylene and hexadecane. BATH assay was performed in TSBYE using xylene and hexadecane, following a method described by Prachaiyo, *et. al.* (177)

Strain	Bacterial Adherence to Hydrocarbon	
	Xylene	Hexadecane
LM21	0.758 ± 0.011^{ab}	0.846 ± 0.036^{a}
IM55	0.715 ± 0.004^{b}	0.879 ± 0.015^{a}
IM54	0.731 ± 0.010^{ab}	0.948 ± 0.074^{a}
IM53	0.783 ± 0.027^{a}	0.868 ± 0.004^{a}

Note: Values are obtained by calculating the average of three replicates followed by standard error of the mean. Characters indicated significant differences within a column when tested with Tukey's HSD at 95% confidence level ($p \leq 0.05$).

Table 5.2. Cell surface charge. Zeta Potential of *L. monocytogenes* wild type strain (LM21) and the 3 deletion mutants (IM55, IM54 and IM53), was measured phosphate buffer saline pH 5.8, 7.0 and 8.0.

Strain	Zeta Potential (mV)		
	pH 5.8	pH 7.0	pH 8.0
LM21	-35.1 ± 0.50	-37.9 ± 0.47	-37.8 ± 0.23
IM55	-36.8 ± 0.15	-38.3 ± 0.23	-37.3 ± 0.35
IM54	-36.6 ± 0.35	-37.8 ± 0.05	-37.4 ± 0.50
IM53	-36.9 ± 0.35	-37.9 ± 0.18	-37.5 ± 0.35

The means were tested against F value at 95% confidence level. The result showed that the effect of pH is significant, the effect of strain is insignificant and the interaction between pH and strain is significant. Due to the significance of interaction, and because we are primarily interested in the difference of strain within each pH, the means are sliced by pH, which enables us to determine the difference among strains within each pH. The result showed that the difference among strains was significant at pH 5.8 and non significant at pH 7.0 and 8.0. However, mean separation using Tukey's HSD cannot detect any significant difference among the strains at pH 5.8, thus we consider the difference among strains to be non significant at all pH.

5.6 Complementation of deletion mutants

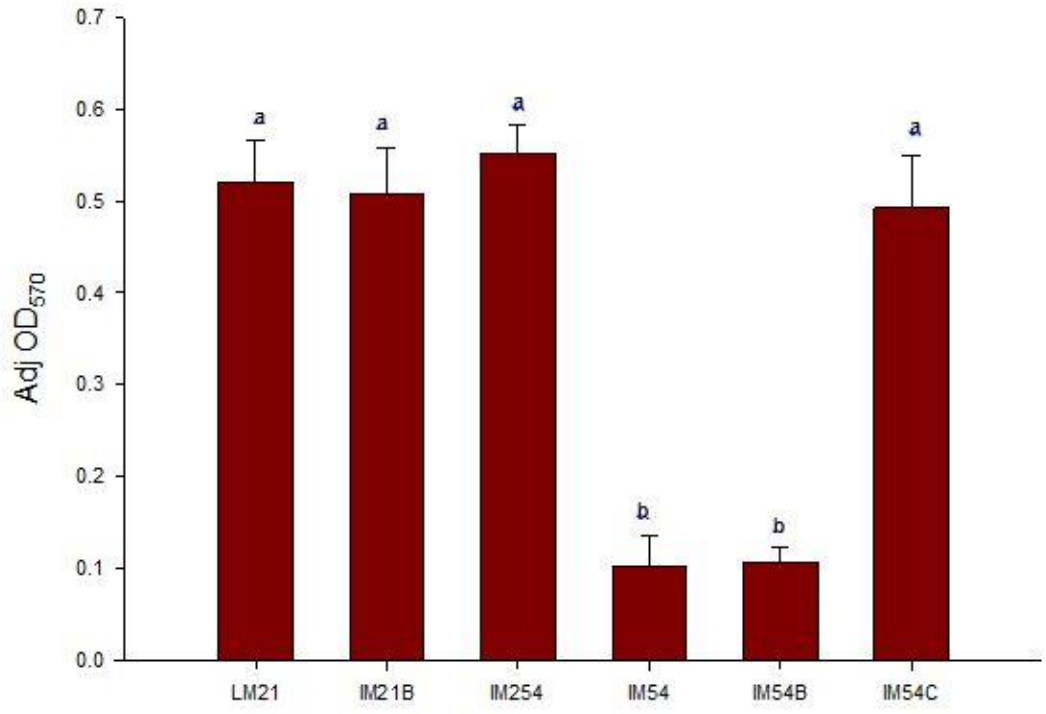
The process of creating a complement for each deletion mutant started by amplifying each gene using primer pairs listed on Table 4.2. Each gene was amplified by PCR reaction and cloned into pIMK2 vector in *E. coli* S17-1, generating recombinant plasmids pTIR255, pTIR254, and pTIR253 (Table 4.3). The pIMK2 plasmid is a site specific listerial integrative vector, which allows constitutive overexpression of genes from the synthetic Phelp promoter. The vector integrates into the $tRNA^{Arg}$ locus, directed by the PSA integrase and has an NcoI site overlapping the ATG of Phelp promoter, thus facilitating a direct, in frame cloning of *Listeria* genes to obtain high level of expression (149, 150).

Each of the recombinant plasmids was then moved into wild type strain and each deletion mutant strains by means of cell conjugation in the following combinations: (i) blank pIMK2 vector into wild type strain and all mutant strains, and (ii) each recombinant plasmid into wild type strain and its respective mutant strain. Strains generated during this process is listed on Table 4.1. Confirmation of complementation was done by means of phenotype analysis, namely biofilm formation on a PVC 96-well plate.

After series of conjugation experiment, we were able to confirm that the insertion of both blank vector and the complement of *lmo2554* gene via pTIR254 into wild type strain (generating strains IM21B and IM254, respectively) did not affect biofilm formation, and that insertion of blank vector into IM54 (generating strain IM54B) gave comparable biofilm formation with IM54. We were also able to confirm that bringing back the complement of *lmo2554* gene into strain IM54 restored the strain's ability to form biofilm on a PVC well plate (Figure 5.6).

Insertion of the complement of *lmo2555* gene via pTIR255 into strain IM55 did not restore the strain's ability to form biofilm on a PVC well plate, which is an indication that the complementation is unsuccessful. We suspected this might be because the deletion of the gene causes polar mutation which affects the expression of the downstream genes along the operon. If polar mutation does take place and the biofim formation ability is not restored when the gene was inserted, this means biofim formation requires more than *lmo2555* gene to take place. Altogether, this lead us to believe that this particular gene is not important in biofilm formation.

Insertion of the complement of *lmo2553* gene via pTIR253 into strain IM53 also did not restore the strain's ability to form biofilm on a PVC well plate. If our suspicion is correct that the *lmo2555-lmo2554-lmo2553* operon is a polycistronic mRNA, and the deletion causes non polar mutation, then the fact that insertion of *lmo2553* back into IM53 strain did not bring back the strain's biofilm formation ability may lead us to believe that the *lmo2553* gene is not important in this aspect.



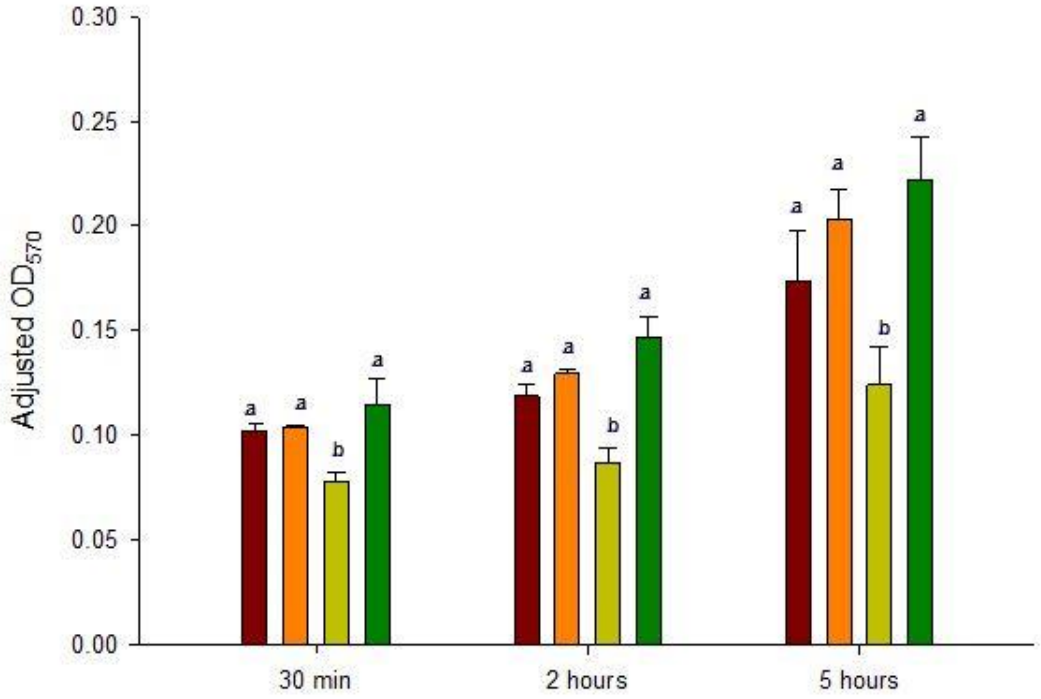
Note: Characters indicated significant differences tested with Tukey's HSD at 95% confidence level or p value ≤ 0.05)

Figure 5.6. Microtiterplate biofilm assessment of *L. monocytogenes* wild type strain (LM21), positive controls (IM21B and IM254), IM54 and IM54B mutants and its complemented strain (IM54C). Assay was performed on a 96-well PVC plate, according to a method developed by Djordjevic, *et. al*. (175). Cells were grown overnight in TSBYE at 32ºC and mixed with Modified Welshimer's Broth (176) at 0.1% concentration. Plates were incubated at 32ºC for approximately 48 hours. Wells were washed with sterile distilled water, stained with 0.1% crystal violet for 45 minutes and de-stained with ethanol for 1 hour. Absorbance was measured using a microtiter plate reader at 570nm.

The initial attachment assay was performed using the mutant strain IM54B, wildtype positive controls (IM21B and IM254) and its complemented strain (IM54C). This experiment was performed in three repetitions, the OD measurement was averaged and plotted on a graph shown on Figure 5.7. Statistical analysis showed that there is a significant difference among time points and among the four different strains. Tukey's HSD analysis at 95% confidence level showed that in all three time points, the four different strains showed similar trends, where the mutant strain IM54B has a significantly lower OD measurement compared to the other three strains (IM21B, IM254 and IM54C). Statistical analysis also showed that the complemented strain (IM54C) did not show significant difference in OD measurement when compared to its two positive controls (IM21B and IM254). This result showed that the complementation of *lmo2554* restored the cell's initial attachment ability, which eventually affect the cell's ability to stick to a PVC surface and form biofilm.

5.7 LTA expression using dot blot immunoassay

Webb, *et. al*. (144) have shown previously that the deletion of *lafA* and *lafB* lead to a drastic reduction in the total amount of lipoteichoic acid produced in *L. monocytogenes* strain 10403S. This leads us to hypothesize that we might see the same phenomena in *L. monocytogenes* strain ScottA. In order to confirm that hypothesis, we performed a dot blot immunoassay, which is a semi quantitative way to assess the amount of lipoteichoic acid in the cell surface. Cells were transferred to a nitrocellulose membrane by a water vacuum. The presence of lipoteichoic acid was determined using a mouse monoclonal antibody specific for LTA from all serotypes of *L. monocytogenes*. Detection of the bound antibody was performed using an Opti-4CN Goat-anti-Mouse antibody with the Goat-anti-Mouse-HRP conjugated secondary antibody. Colorimetric detection was performed using an Opti-4CN substrate and diluent that comes with the Opti-4CN detection kit.



Note: Characters indicated significant differences tested with Tukey's HSD at 95% confidence level or p value ≤ 0.05)

Figure 5.7. Initial attachment assay for *L. monocytogenes* IM54 mutant strain with its complemented strain (IM54C) and positive controls (IM21B and IM254). Overnight grown cells are pelleted and OD_{600} was adjusted to 1.0. One percent inoculum was added to MWB supplemented with 50 µg/mL Kanamycin, and incubated at 32ºC for 30 minutes, 2 hours and 5 hours. After each time point, plates were washed, stained with 0.1% crystal violet, and de-stained with ethanol. Absorbance were measured using a microtiter plate reader at 570nm.

IM21B IM254 IM54B IM54C

We used *E. coli* S17-1 as a negative control, where mouse monoclonal antibody should show no color due to the absence of LTA in the cell. In addition to the mutant strains IM54 and IM54B, we used wild type LM21 and IM21B as our positive controls, along with our complemented strain IM54C. The results are shown on Figure 5.8, where the two mutant strain IM54 and IM54B showed a less intense color compared to LM21, IM21B and IM54C, even in the non-diluted samples. The difference in color intensity was more pronounced in the higher dilutions (1:16 and 1:64) before all samples showed no color as the samples become way too diluted for the monoclonal antibody to detect.

5.7 Biofilm Assay under Flowing Condition

Biofilm assay under flow conditions was performed using the mutant strain IM54B, its two wild type vector (IM21B) and complement control (IM254), and its complemented strain (IM54C). Biofilm was grown using a drip flow reactor (DFR) for 48 hours on a stainless steel slides and cell numbers were determined by scraping and plate counts. The cell numbers were averaged and plotted on a graph shown on Figure 5.9.

Analysis of Variance showed that the four strains used are significantly different. Mutant strain IM54B showed a reduced biofilm formation under flow condition by approximately 1 log (90%). Further mean separation using Tukey's HSD showed that IM21B and IM54C were not significantly different, while the mutant strain IM54B was significantly different from its two positive controls (IMCL21B and IM254) and its complemented strain (IM54C). This result showed that the deletion of *lmo2554* reduced the cells' ability to form biofilm under flow condition and that complementation of the gene restored the cells' ability to form biofilm.

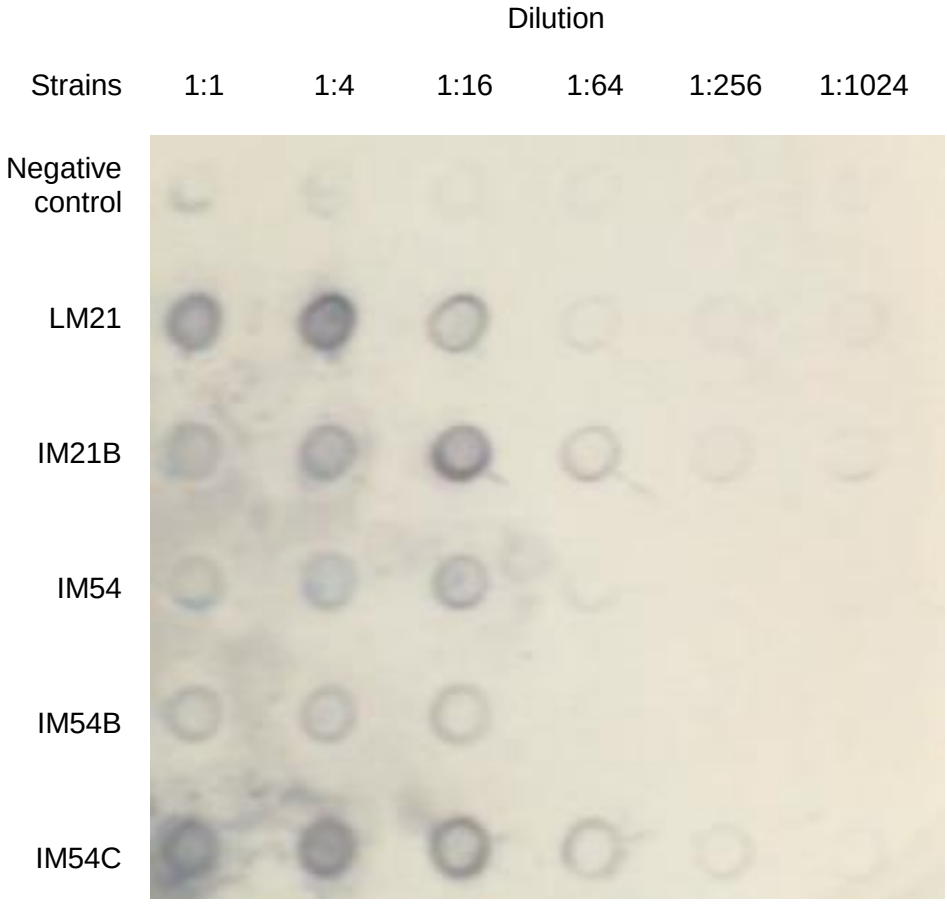
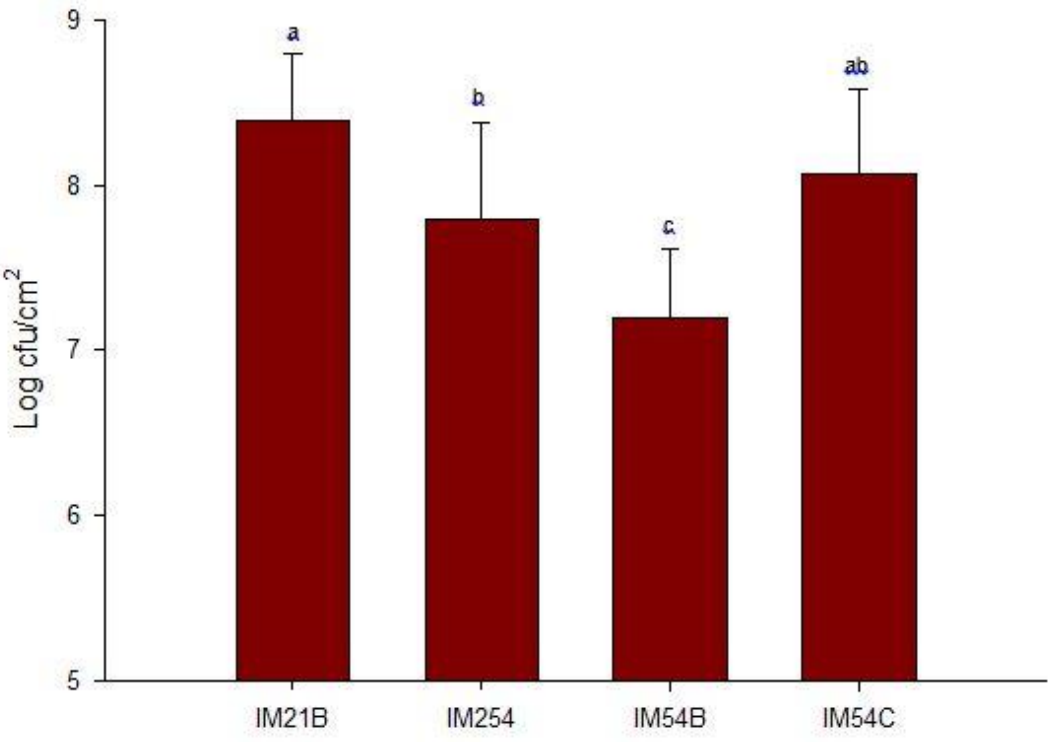


Figure 5.8. Dot blot immunoassay for LTA expression of *L. monocytogenes* wild type, mutant, and complement strains. Assay was performed using a mouse monoclonal antibody specific for LTA from all serotypes of *L. monocytogenes*. Detection of the bound antibody was performed using an Opti-4CN Goat-anti-Mouse antibody (Bio-Rad, Hercules, CA), with the Goat-anti-Mouse-HRP conjugated secondary antibody. Colorimetric detection was performed using an Opti-4CN substrate and diluent that comes with the Opti-4CN detection kit (Bio-Rad, Hercules, CA). *E. coli* S17-1 was used as a negative control.



Note: Characters indicated significant differences tested with Tukey's HSD at 95% confidence level or p value ≤ 0.05)

Figure 5.9. Biofilm formation of IM54B on stainless steel in a drip flow reactor. Mutant strain IM54B, its two positive controls (IM21B and IM254) and its complemented strain (IM54C were grown using drip flow reactor, which consists of four separate channel that holds a single stainless steel plate. Colonies were grown in TSBYE at 32°C overnight. An aliquot of 1mL overnight growth were mixed with 15mL of MWB and incubated for approximately 24 hours at 32°C without flow (batch phase), followed by steady flowing fresh MWB of 0.8mL per minute (continuous phase) for 48 hours. Stainless steel plates were scraped into sterile 0.1% Buffered Peptone Water, serially diluted and plated onto TSAYE. Cell numbers expressed on the graph were obtained from an average of three repetitions.

Analysis of Variance showed that the four strains used are significantly different. Mutant strain IM54B showed a reduced biofilm formation under flow condition by approximately 1 log (90%). Further mean separation using Tukey's HSD showed that IM21B and IM54C were not significantly different, while the mutant strain IM54B was significantly different from its two positive controls (IMCL21B and IM254) and its complemented strain (IM54C). This result showed that the deletion of *lmo2554* reduced the cells' ability to form biofilm under flow condition and that complementation of the gene restored the cells' ability to form biofilm.

5.8 Visualization of *L. monocytogenes* biofilm using confocal microscopy

Biofilms were grown using a drip flow reactor and stained with FM1-43 green biofilm cell stain. Strain IM21B showed a brighter and thicker green color that covers almost the whole field under the microscope (Figure 5.10). Mutant strain IM54B, however, showed a more sparsely located cells. It was relatively difficult to locate cells due to the lower amount of cells present. The complement strain IM54C showed relatively more cells compared to mutant strain. Cells are also not as sparse, although not as bright and not as thick as positive control IM21B. This showed that the deletion of *lmo2554* gene decreases cell density when biofilms were grown on a glass surface and that bringing the gene back restores the cell density in our biofilm.

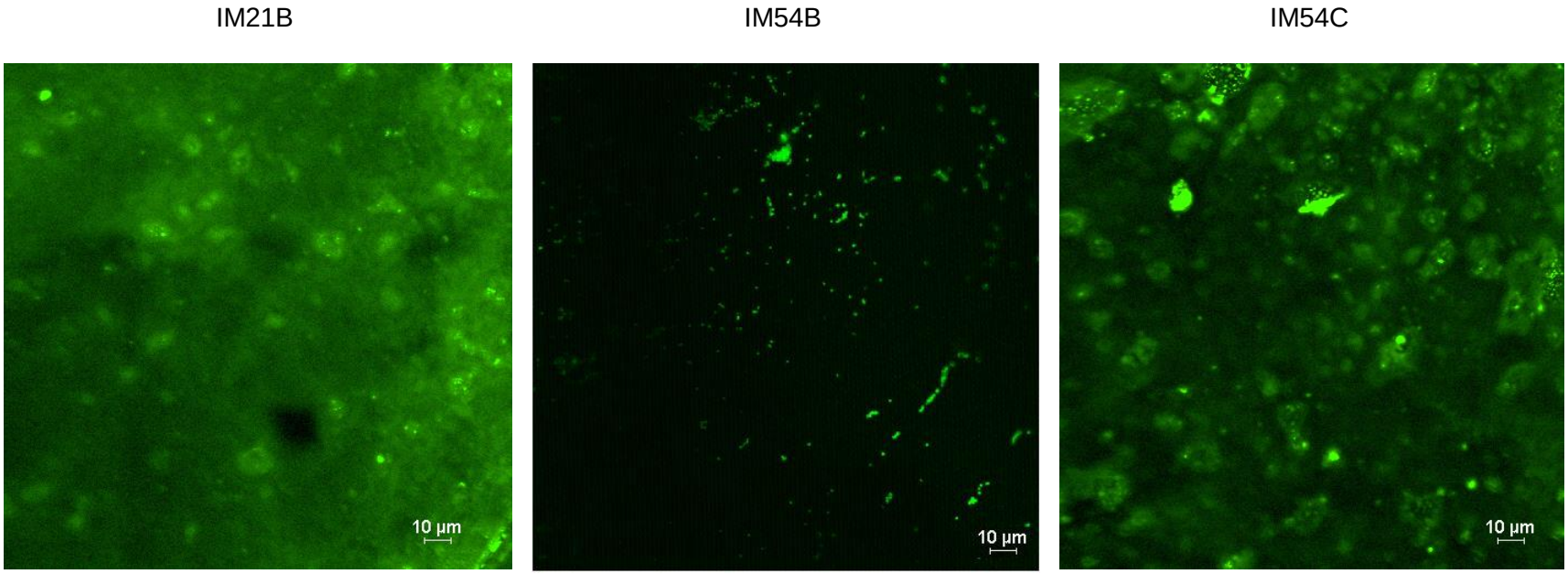


Figure 5.10 Visualization of biofilm cells using confocal microscopy. Biofilms were grown using a drip flow reactor on glass cover slips according to method described in 4.14. Cells were stained with FM1-43 green biofilm stain. Images were taken using Nikon Eclipse 80i confocal microscope system, under green filter (419/515 nm excitation/emission wavelength) with 60x oil immersion lens.

5.9 The influence of lipoteichoic acid upon sensitivity of *L. monocytogenes* to antimicrobial compounds

The resistance of *L. monocytogenes* cells were tested against two ammonium-based disinfectants, namely benzethonium chloride (BZT) and cetylpyridium chloride (CPC), a cationic surfactant lauric arginate (LAE) and a negatively charged component sodium dodecyl sulfate (SDS) on PVC 96-well plates. Cells from 4 different strains, namely the mutant strain (IM54B), its two wild type controls (IM21B and IM254), along with its complement (IM54C) were grown in TSBYE the presence of various concentrations of the tested component. Minimum Inhibitory Concentration (MIC) was defined as the concentration where there was no turbidity present after growth at 32°C for 72 h. Results were recorded in Table 5.3.

The results showed that between the two QAC components, *L. monocytogenes* have higher MIC for BZT compared to CPC. After 72 hours of incubation, IM21B, IM254, IM54B, and IM54C showed MIC of 4, 4, 3, and 4ppm, respectively for BZT, and 2, 2, 1, and 2ppm, respectively for CPC. The results indicate that the loss of LTA in IM54B lowered the ability of *L. monocytogenes* to grow in the presence of QAC.

L. monocytogenes also showed different sensitivity to cationic antimicrobial LAE. The two positive controls IM21B and IM254, along with complement strain IM54C showed MIC at 31ppm, while mutant strain IM54B showed MIC at 20ppm. Similar trend was also observed when tested against anionic antimicrobial SDS. The two positive controlsIM21B and IM254, along with the complement strain IM54C showed a much higher resistance (up to 6 times higher) to SDS with MIC at 240ppm, 238ppm and 240ppm, respectively, compared to the mutant strain IM54B at 41ppm.

Table 5.3. Minimum Inhibitory Concentration (MIC) Assay. Strains IM21B, IM254, IM54B and IM54C were treated with quarternary-based ammonium compounds (BZT and CPC), Lauric Arginate (LAE), and Sodium Dodecyl Sulfate (SDS), grown in TSBYE, incubated for 72 hours at 32^{o}C.

Strain	Minimum Inhibitory Concentration (MIC) in ppm			
	BZT	CPC	LAE	SDS
IM21B	4	2	31	240
IM254	4	2	31	238
IM54B	3	1	20	41
IM54C	4	2	31	240

5.10 The influence of lipoteichoic acid (LTA) on growth of *L. monocytogenes* at different temperatures

We observed slower growth in all our control plates incubated at lower temperature (Figure 5.11). At 32^{o}C and 37^{o}C, we observed similar growth rates for all three strains we used, namely IM21B (positive control), IM54B (mutant strain), and IM54C (complement strain). At 20^{o}C, growth seems to be slightly affected, where all three strains seemed to grow slower. This is clear by the fewer number of colonies on the plate on day 2, however by day 5 and 7 at 20 °C, similar cell growth was observed.

The effect of temperature is more pronounced when plate was incubated at 4^{o}C, where we started seeing growth of IM21B and IM54C on day 7 and no growth on day 2 and day 5. We extend incubation to 14 days and we were able to confirm the slower growth at 4^{o}C by observing more growth of IM21B and IM54C after a 14-day incubation. Our mutant strain (IM54B) seem to grow fine when incubated at 32^{o}C and 37^{o}C, where it showed comparable growth with our positive control (IM21B) and its complement strain (IM54C). At 20°C, the growth rate, of IM54C appeared to be slower, and it did not grow when incubated at 4^{o}C even after a 14-day incubation.

We also performed cell morphology observation for all strains, both wild type LM21 and mutant strains IM21B, IM54, IM54B, and IM54C. Cells were grown in TSBYE at 4^{o}C, 20^{o}C, 32^{o}C, and 37^{o}C. After visualization under microscope with DIC filter, we saw no significant difference in cell morphology between our wild type and mutant strains (results not shown).

5.11 The influence of lipoteichoic acid (LTA) on growth of *L. monocytogenes* under reduced water activity

We assessed the influence of LTA in the survival of *L. monocytogenes* exposed to osmotic stress at different temperature by growing cells on TSAYE plates with different a_w

and incubating them at different temperature. We used three different humectants, namely NaCl, sucrose and glycerol to bring the a_w down to 0.98, 0.95, 0.93 and 0.90, and incubating plates at 4°C, 20°C, 32°C, and 37°C. The result was tabulated in the form of figures (Figure 5.11 to Figure 5.14) and tables (Table 5.4 to 5.7).

When we compared our mutant strain IM54B with its positive control IM21B and IM54C, we observed different sensitivity to different humectants at the lower temperatures (20°C and 4°C). However, an exception to this statement is when cells are exposed to NaCl. We observed that all three strains did not seem to show different sensitivity to NaCl across all a_w at all temperatures, which is an indication that the absence of LTA did not seem to affect the cells' tolerance against NaCl, specifically at lower a_w (0.95 and 0.93) when incubated at higher temperature (32°C and 37°C).

We also noticed that the three different strains greater sensitivity to reduced a_w when sucrose was the humectant. This is seen all across all temperature at lower a_w (0.95 and 0.93). However, at 4°C, the increased sensitivity of the three different strains to sucrose is noticeable in all a_w (Figure 5.11 and Table 5.4). Overall, glycerol seems to be the less harsh to all strains. This is obvious when comparing growth at a_w 0.90 when a_w was lowered using NaCl and glycerol. At all temperatures, we were able to see growth of our control (IM21B) when glycerol was used as humectant, but not with NaCl.

When we compared our mutant strain IM54B with its positive control IM21B and IM54C, we observed different sensitivity to different humectants at lower temperature (20°C and 4°C). However, an exception to this statement is when cells are exposed to NaCl. We observed that all three strains did not seem to show different sensitivity to NaCl across all a_w at all temperatures, which is an indication that the absence of LTA did not seem to affect the cells' tolerance against NaCl, specifically at lower a_w (0.95 and 0.93) when incubated at higher temperature (32°C and 37°C).

We also noticed that the three different strains showed different sensitivity when exposed to sucrose. This is seen all across all temperature at lower a_w (0.95 and 0.93). However, at 4^{o}C, the different sensitivity of the three different strains to sucrose is noticeable in all a_w (Figure 5.12 and Table 5.4).

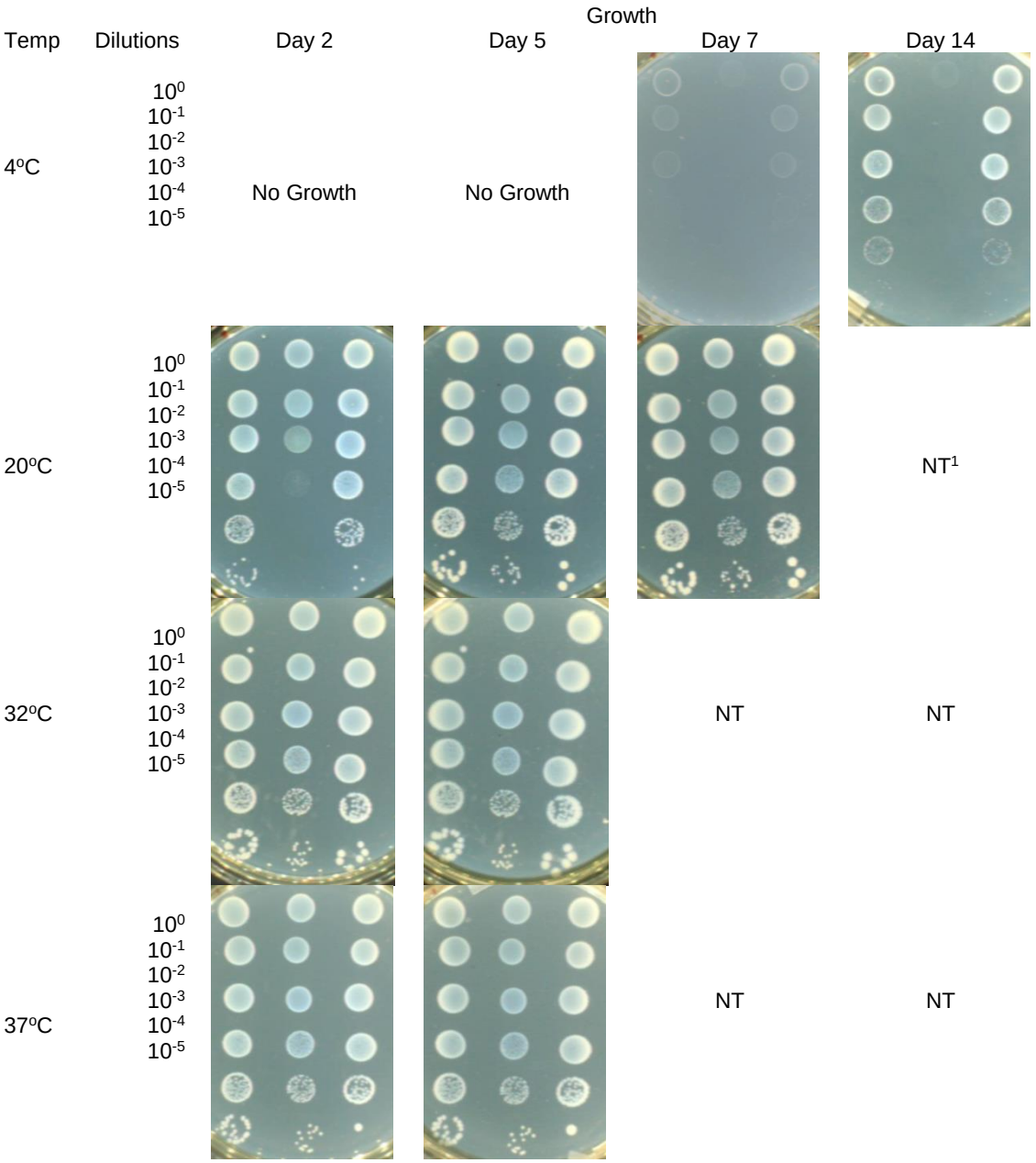


Figure 5.11. Growth on TSBYE plates at different temperatures. An aliquot of 10µL and its serial dilutions was inoculated onto TSAYE plates. Plates were incubated at 4ºC, 20ºC, 32ºC, and 37ºC.

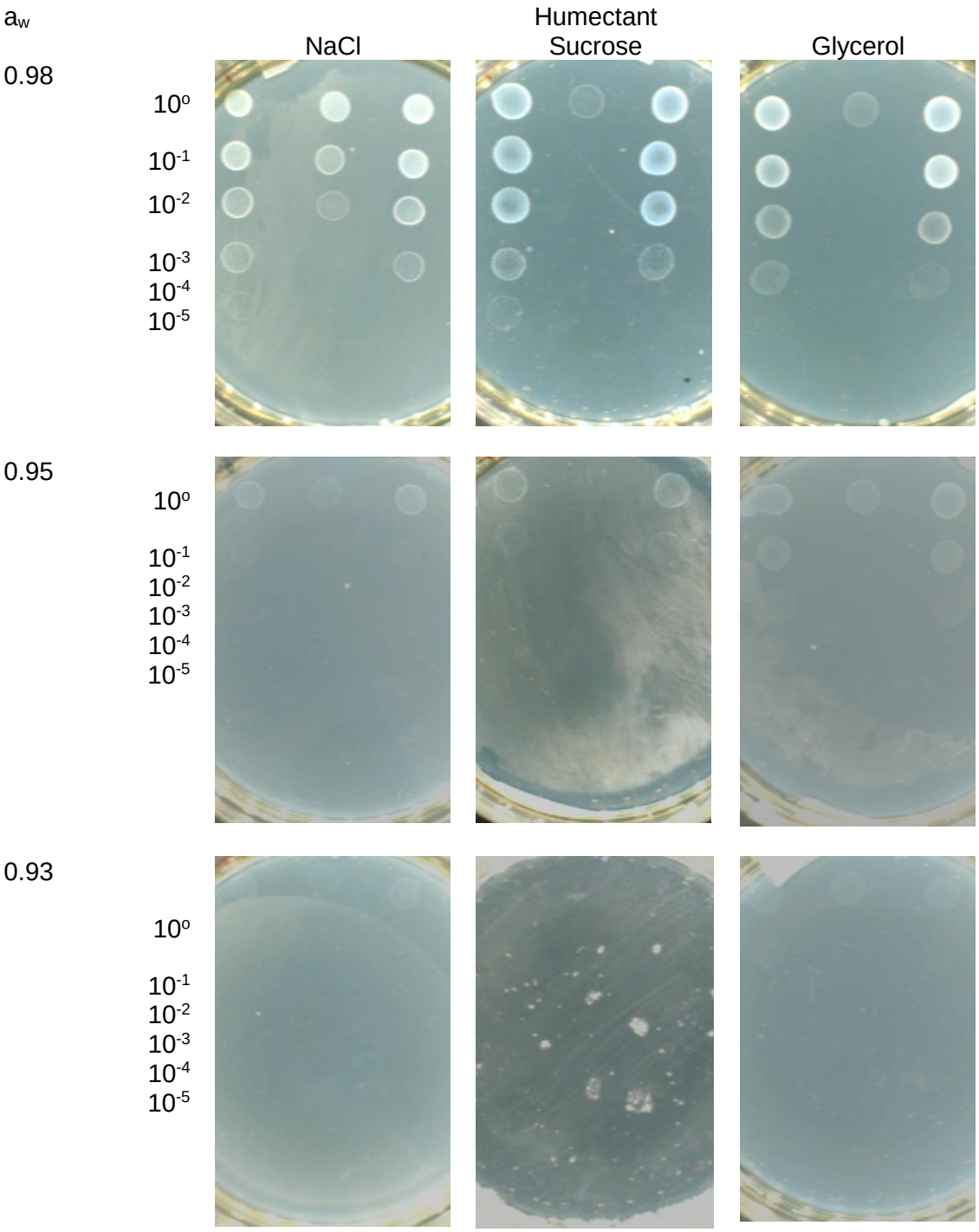


Figure 5.12. Growth at 4ºC in the presence of different humectants. An aliquot of 10µL and its serial dilutions was inoculated onto plates with a_w altered with different humectant; NaCl, sucrose, and glycerol. Plates were incubated for 14 days.

Table 5.4. Influence of humectant on growth at 4^{o}C. An aliquot of 10µL and its serial dilutions was inoculated onto plates with a_w altered with different humectant; NaCl, sucrose, and glycerol. Plates were incubated for 14 days.

a_w	Humectant	Strain	Dilutions[1]					
			10^{o}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Control	N/A	IM21B	++++[2]	++++	++++	+++	+++	+
		IM54B	+	-	-	-	-	-
		IM54C	++++	++++	++++	+++	+++	-
a_w 0.98	NaCl	IM21B	++++	++++	+++	+++	+	-
		IM54B	++++	+++	+++	-	-	-
		IM54C	++++	++++	+++	+++	-	-
	Sucrose	IM21B	++++	++++	++++	+++	++	-
		IM54B	+	-	-	-	-	-
		IM54C	++++	++++	++++	+++	-	-
	Glycerol	IM21B	++++	++++	+++	+++	-	-
		IM54B	+	-	-	-	-	-
		IM54C	++++	++++	+++	+++	-	-
a_w 0.95	NaCl	IM21B	++	+	-	-	-	-
		IM54B	++	+	-	-	-	-
		IM54C	++	+	-	-	-	-
	Sucrose	IM21B	++	+	-	-	-	-
		IM54B	-	-	-	-	-	-
		IM54C	++	+	-	-	-	-
	Glycerol	IM21B	++	++	+	-	-	-
		IM54B	+	-	-	-	-	-
		IM54C	++	++	+	-	-	-
a_w 0.93	NaCl	IM21B	+	-	-	-	-	-
		IM54B	+	-	-	-	-	-
		IM54C	+	-	-	-	-	-
	Sucrose	IM21B	-	-	-	-	-	-
		IM54B	-	-	-	-	-	-
		IM54C	-	-	-	-	-	-
	Glycerol	IM21B	+	-	-	-	-	-
		IM54B	+	-	-	-	-	-
		IM54C	+	-	-	-	-	-
a_w 0.90	NaCl	IM21B	-	-	-	-	-	-
		IM54B	-	-	-	-	-	-
		IM54C	-	-	-	-	-	-
	Glycerol	IM21B	+	-	-	-	-	-
		IM54B	+	-	-	-	-	-
		IM54C	+	-	-	-	-	-

[1]Initial inoculation in 10µL drop at 10^{o} dilution is as follows: $2.1x10^{6}$, $8x10^{5}$, and $1.6x10^{6}$ cfu per drop for IM21B, IM54B, and IM54C, respectively.

[2]++++: confluent drop, heavy growth ++++ confluent drop, lighter growth
++ confluent drop, light growth + confluent drop, colonies feasible - no growth

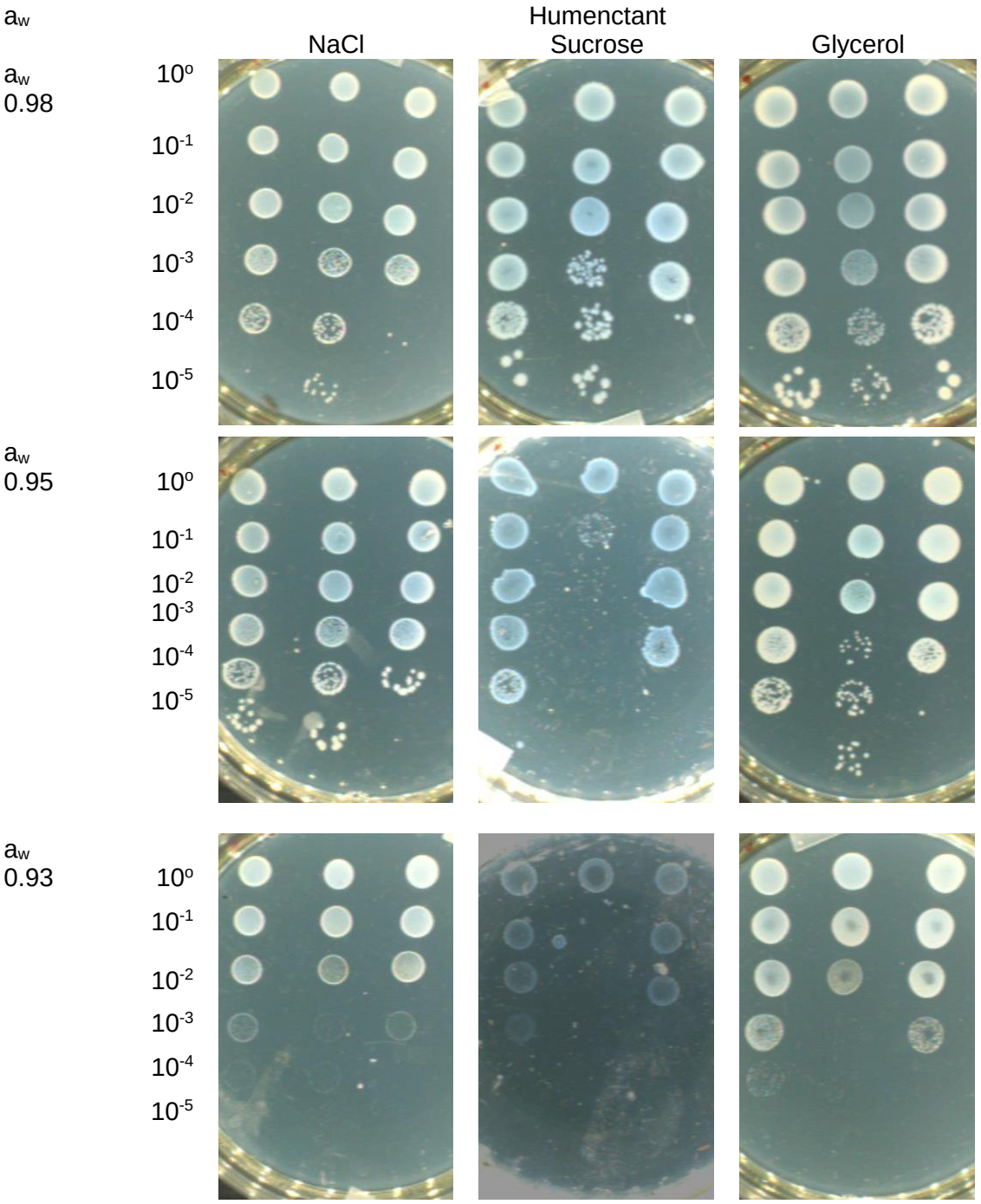


Figure 5.13. Growth at 20°C in the presence of different humectants. An aliquot of 10µL and its serial dilutions was inoculated onto plates with a_w altered with different humectant; NaCl, sucrose, and glycerol. Plates were incubated for 7 days.

Table 5.5. Influence of humectant on growth at 20°C. An aliquot of 10µL and its serial dilutions was inoculated onto plates with a_w altered with different humectant; NaCl, sucrose, and glycerol. Plates were incubated for 7 days.

a_w	Humectant	Strain	Dilutions[1]					
			10^o	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Control	N/A	IM21B	++++[2]	++++	++++	++++	+++	++
		IM54B	++++	++++	++++	+++	+++	++
		IM54C	++++	++++	++++	++++	+++	++
a_w 0.98	NaCl	IM21B	++++	++++	++++	+++	+++	-
		IM54B	++++	++++	++++	+++	+++	++
		IM54C	++++	++++	++++	+++	+	-
	Sucrose	IM21B	++++	++++	++++	++++	+++	+
		IM54B	++++	++++	++++	+++	+++	+
		IM54C	++++	++++	++++	+++	+	-
	Glycerol	IM21B	++++	++++	++++	++++	+++	++
		IM54B	++++	++++	++++	+++	+++	++
		IM54C	++++	++++	++++	++++	+++	++
a_w 0.95	NaCl	IM21B	++++	++++	++++	+++	+++	++
		IM54B	++++	++++	++++	+++	+++	++
		IM54C	++++	++++	++++	+++	++	-
	Sucrose	IM21B	++++	++++	++++	+++	++	-
		IM54B	++++	+	-	-	-	-
		IM54C	++++	++++	++++	+++	-	-
	Glycerol	IM21B	++++	++++	++++	++++	+++	-
		IM54B	++++	++++	+++	++	++	++
		IM54C	++++	++++	++++	+++	+	-
a_w 0.93	NaCl	IM21B	++++	++++	+++	++	-	-
		IM54B	++++	++++	+++	-	-	-
		IM54C	++++	++++	+++	+	-	-
	Sucrose	IM21B	++	++	+	+	-	-
		IM54B	+	-	-	-	-	-
		IM54C	++	++	+	+	-	-
	Glycerol	IM21B	++++	++++	++++	+++	+	-
		IM54B	++++	++++	+++	-	-	-
		IM54C	++++	++++	++++	+++	-	-
a_w 0.90	NaCl	IM21B	-	-	-	-	-	-
		IM54B	-	-	-	-	-	-
		IM54C	-	-	-	-	-	-
	Glycerol	IM21B	++	+	-	-	-	-
		IM54B	+	-	-	-	-	-
		IM54C	++	+	-	-	-	-

[1]Initial inoculation in 10µL drop at 10^o dilution is as follows: $2.1x10^6$, $8x10^5$, and $1.6x10^6$ cfu per drop for IM21B, IM54B, and IM54C, respectively.

[2]++++: confluent drop, heavy growth ++++ confluent drop, lighter growth
++ confluent drop, light growth + confluent drop, colonies feasible - no growth

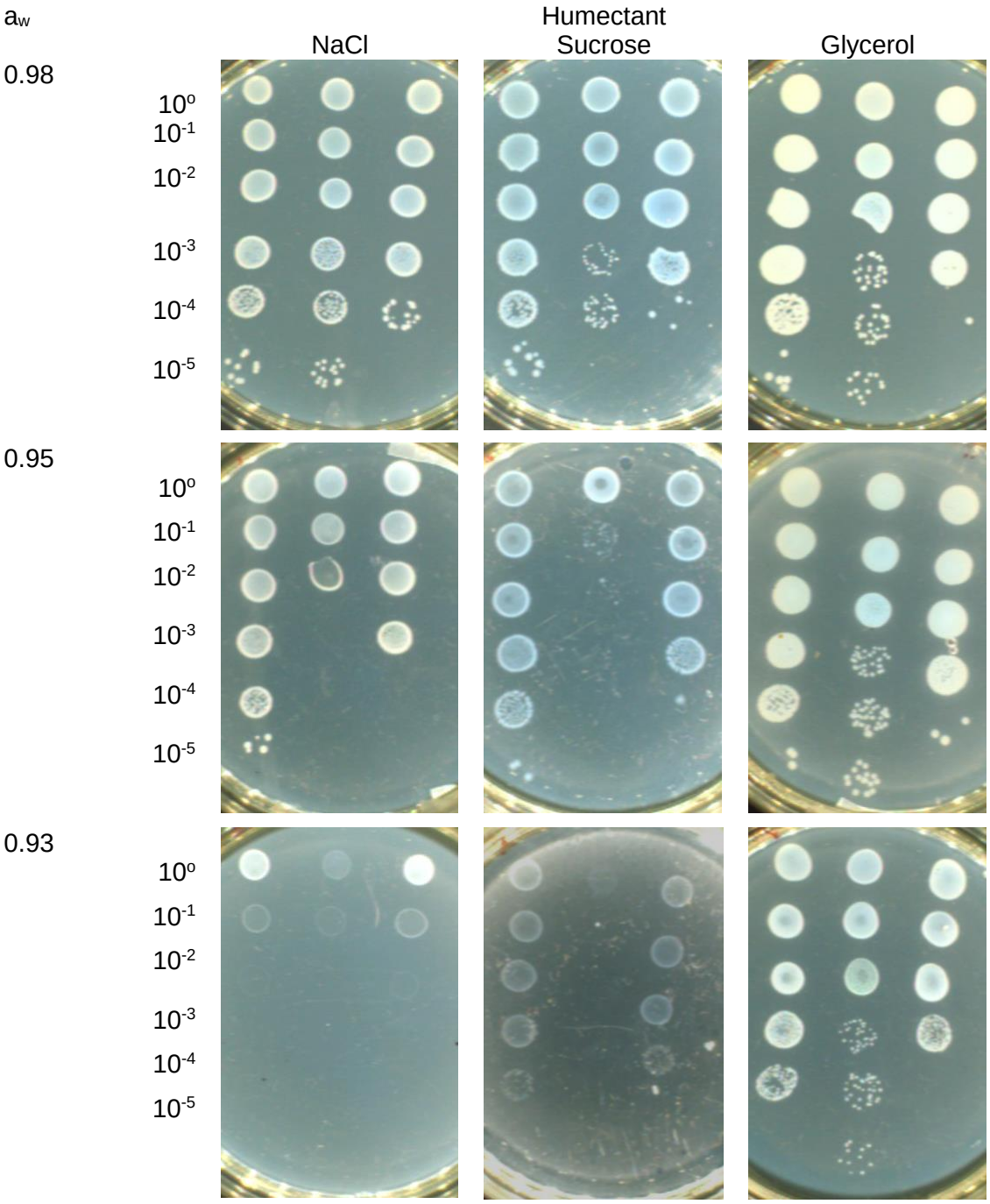


Figure 5.14. Growth at 32ºC in the presence of different humectants. An aliquot of 10µL and its serial dilutions was inoculated onto plates with a_w altered with different humectant; NaCl, sucrose, and glycerol. Plates were incubated for 5 days.

Table 5.6. Influence of humectant on growth at 32°C. An aliquot of 10µL and its serial dilutions was inoculated onto plates with a_w altered with different humectant; NaCl, sucrose, and glycerol. Plates were incubated for 5 days.

a_w	Humectant	Strain	Dilutions[1]					
			10^o	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Control	N/A	IM21B	++++[2]	++++	++++	++++	+++	++
		IM54B	++++	++++	++++	+++	++	+
		IM54C	++++	++++	++++	++++	+++	++
a_w 0.98	NaCl	IM21B	++++	++++	++++	+++	++	+
		IM54B	++++	++++	++++	+++	++	+
		IM54C	++++	++++	++++	+++	+	-
	Sucrose	IM21B	++++	++++	++++	+++	++	+
		IM54B	++++	++++	++++	+	+	-
		IM54C	++++	++++	++++	+++	+	-
	Glycerol	IM21B	++++	++++	++++	++++	+	-
		IM54B	++++	++++	+++	+	+	+
		IM54C	++++	++++	++++	++++	-	-
a_w 0.95	NaCl	IM21B	++++	++++	++++	+++	++	+
		IM54B	++++	++++	+++	-	-	-
		IM54C	++++	++++	++++	+++	-	-
	Sucrose	IM21B	++++	++++	++++	+++	++	-
		IM54B	++++	+	-	-	-	-
		IM54C	++++	++++	++++	+++	-	-
	Glycerol	IM21B	++++	++++	++++	++++	+++	+
		IM54B	++++	++++	++++	++	++	++
		IM54C	++++	++++	++++	++++	+	-
a_w 0.93	NaCl	IM21B	++++	++	+	-	-	-
		IM54B	+++	++	-	-	-	-
		IM54C	++++	++	+	-	-	-
	Sucrose	IM21B	++	++	++	++	+	-
		IM54B	+	-	-	-	-	-
		IM54C	++	++	++	+	-	-
	Glycerol	IM21B	++++	++++	++++	+++	++	-
		IM54B	++++	+++	+++	++	++	+
		IM54C	++++	++++	++++	+++	-	-
a_w 0.90	NaCl	IM21B	-	-	-	-	-	-
		IM54B	-	-	-	-	-	-
		IM54C	-	-	-	-	-	-
	Glycerol	IM21B	++	+	-	-	-	-
		IM54B	+	-	-	-	-	-
		IM54C	++	+	-	-	-	-

[1]Initial inoculation in 10µL drop at 10^o dilution is as follows: $2.1x10^6$, $8x10^5$, and $1.6x10^6$ cfu per drop for IM21B, IM54B, and IM54C, respectively.

[2]++++: confluent drop, heavy growth +++ confluent drop, lighter growth
++ confluent drop, light growth + confluent drop, colonies feasible - no growth

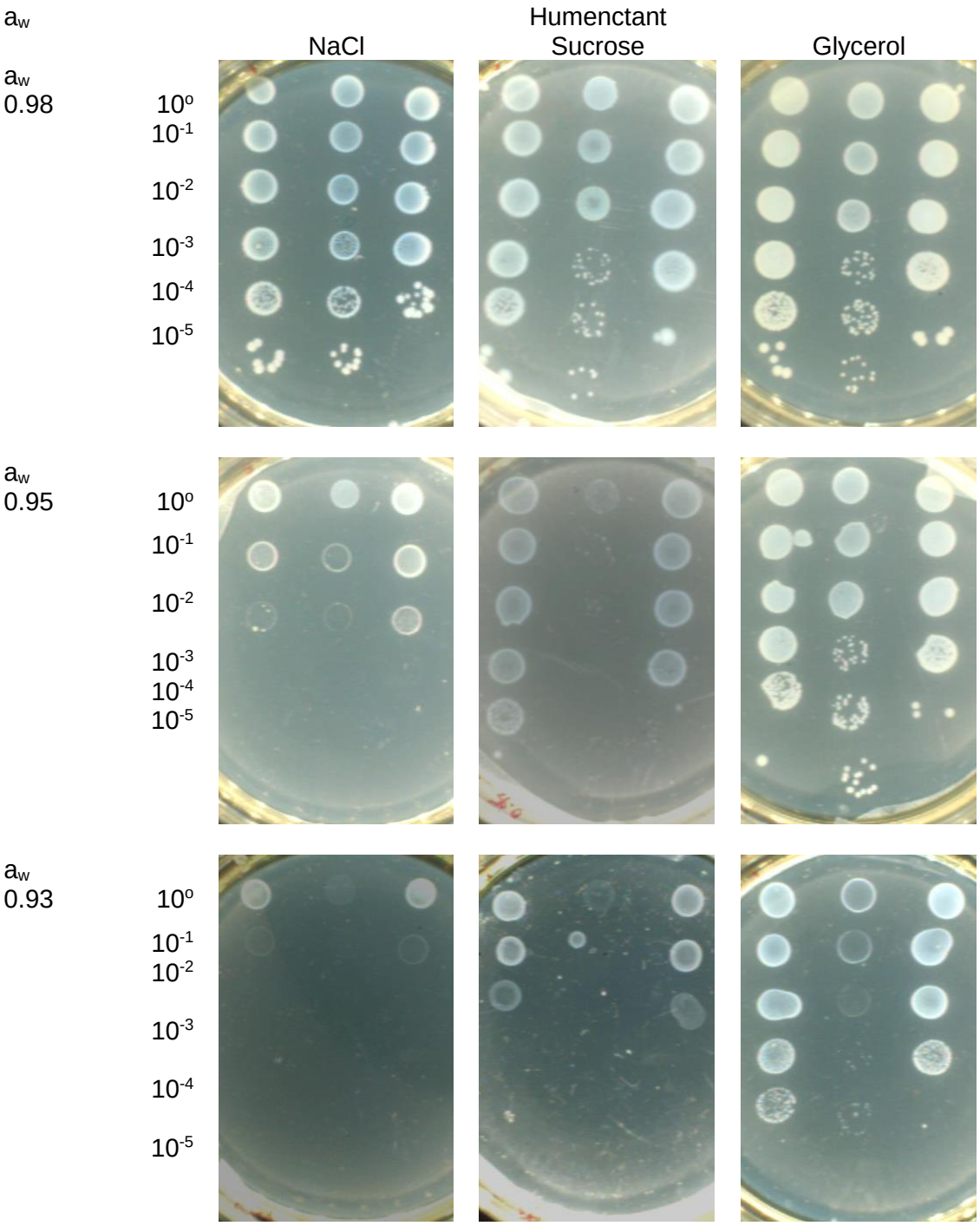


Figure 5.15. Growth at 37ºC in the presence of different humectants. An aliquot of 10µL and its serial dilutions was inoculated onto plates with a_w altered with different humectant; NaCl, sucrose, and glycerol. Plates were incubated for 5 days.

Table 5.7. Influence of humectant on growth at 37°C. An aliquot of 10µL and its serial dilutions was inoculated onto plates with a_w altered with different humectant; NaCl, sucrose, and glycerol. Plates were incubated for 5 days.

a_w	Humectant	Strain	Dilutions[1]					
			10^o	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Control	N/A	IM21B	++++[2]	++++	++++	++++	+++	++
		IM54B	++++	++++	++++	+++	+++	++
		IM54C	++++	++++	++++	++++	+++	++
a_w 0.98	NaCl	IM21B	++++	++++	++++	+++	+++	++
		IM54B	++++	++++	++++	+++	+++	++
		IM54C	++++	++++	++++	+++	++	-
	Sucrose	IM21B	++++	++++	++++	+++	+++	+
		IM54B	+++	+++	+++	++	++	+
		IM54C	++++	++++	++++	+++	+	-
	Glycerol	IM21B	++++	++++	++++	++++	+++	++
		IM54B	+++	+++	+++	++	++	++
		IM54C	++++	++++	++++	+++	++	-
a_w 0.95	NaCl	IM21B	++++	+++	+	-	-	-
		IM54B	+++	+	+	-	-	-
		IM54C	++++	+++	+++	-	-	-
	Sucrose	IM21B	++++	++++	++++	+++	+++	-
		IM54B	++	-	-	-	-	-
		IM54C	++++	++++	++++	+++	-	-
	Glycerol	IM21B	++++	++++	++++	++++	+++	+
		IM54B	++++	++++	+++	++	++	++
		IM54C	++++	++++	++++	+++	+	-
a_w 0.93	NaCl	IM21B	++	+	-	-	-	-
		IM54B	+	-	-	-	-	-
		IM54C	++	+	-	-	-	-
	Sucrose	IM21B	++++	+++	++	-	-	-
		IM54B	+	-	-	-	-	-
		IM54C	++++	+++	++	-	-	-
	Glycerol	IM21B	++++	++++	++++	+++	+++	-
		IM54B	+++	+	-	-	-	-
		IM54C	++++	++++	++++	+++	-	-
a_w 0.90	NaCl	IM21B	+	-	-	-	-	-
		IM54B	+	-	-	-	-	-
		IM54C	+	-	-	-	-	-
	Glycerol	IM21B	++	+	-	-	-	-
		IM54B	+	-	-	-	-	-
		IM54C	++	+	-	-	-	-

[1]Initial inoculation in 10µL drop at 10^o dilution is as follows: $2.1x10^6$, $8x10^5$, and $1.6x10^6$ cfu per drop for IM21B, IM54B, and IM54C, respectively.

[2]++++: confluent drop, heavy growth ++++ confluent drop, lighter growth
++ confluent drop, light growth + confluent drop, colonies feasible - no growth

CHAPTER VI

DISCUSSION

6.1 Creation of In-Frame Deletion Mutants

Previous results in our laboratory (166) were able to identify 24 distinct reduced biofilm formation (RBF) mutants, with reduced abilities to form biofilm to varying degrees. This suggests that different genes contribute diversely at a varying degree in biofilm formation. The 24 mutants were divided into three different groups based on their ability to form biofilm on a PVC surface. Group I consisted of 6 members whose biofilm formation abilities were reduced more than 40% compared to that of wild type strain. Within Group I, the transposon disrupted genes included *lmo2555* and *lmo2554*. Lmo2555 protein, or known as LTA anchor formation protein A (LafA), encodes glycosyltransferase specific for UDP glucose and responsible for Glc-DAG synthesis. Lmo2554 protein, or known as LafB, encodes UDP-galactose and responsible for Gal-Glc-DAG synthesis. Altogether this lead hypothesize that either one or both of these genes plays a critical role in biofilm formation. However, since transposon mutants may cause polar effects, in-frame deletion mutants of each of these genes was generated in order to further understand on the role of LTA in *L. monocytogenes* biofilm formation.

L. monocytogenes serovar 4b, strain ScottA served as our wild type strain throughout the course of this research project. This particular strain is a clinical isolate from the 1983 listeriosis outbreak in Massachusetts (179). The genome has been successfully sequenced, which gives a total of 3,021,822 bp, 2,953 predicted open reading frames (ORF), at least 65 tRNA genes, two pseudo-tRNAs, six copies of 16S-23S-5S operons, and two prophage sequences (180).

Creation of in-frame deletion mutant was performed in order to further characterize the importance of each gene in the *lmo2555–lmo2554–lmo2553* operon. The gene map of the operon can be seen in Figure 6.1. In *L. monocytogenes* strain EGD-e, it is designated as polycistronic operon number 458 and is composed of four genes, namely *lmo2555, lmo2554, lmo2553* and murZ, with a terminator right after *lmo2553* (181). Upstream of *lmo2555*, the presence of another gene *lmo2556* has been confirmed and recognized as similar to fructose-1.6-bisphosphate aldolase (fbaA).

The construction of primers during the creation of deletion fragments for *lmo2555* and *lmo2554* was based on sequences from strain EGD-e, and *lmo2553* was based on strain Scott A. The upstream and downstream sections were amplified ligated, then cloned into temperature-sensitive integrative shuttle vector pKSV7 (169), creating recombinant plasmids pTIR755, pTIR754, and pTIR753, carrying each deletion fragment *lmo2555*, *lmo2554*, and *lmo2553*, respectively. Recombinant plasmids were allowed to replicate in *E. coli*. Previous research has shown that pKSV7 plasmid replicates at high copy number in *E. coli* and allows the identification inserts in the polylinker cluster, and thus should be useful for other kinds of genetic manipulation in Gram positive bacteria, such as *B. subtilis* (169) and *L. monocytogenes* (182). Recombinant plasmids were electroporated into *E. coli* DH5α electro competent cells. Insertion of deletion fragments into the plasmid was indicated firstly by the presence of white colonies on the agar plate. Further confirmation was performed by double digestion of the recombinant plasmid with XbaI and EcoRI, followed by gel electrophoresis.

Introduction of recombinant plasmid into *L. monocytogenes* wild type strain was performed using electroporation into *L. monocytogenes* electro competent cells. In our hands, *L. monocytogenes* strain LM21 was proven to be poorly transformable. This is consistent with results shown by others (170, 172, 183).

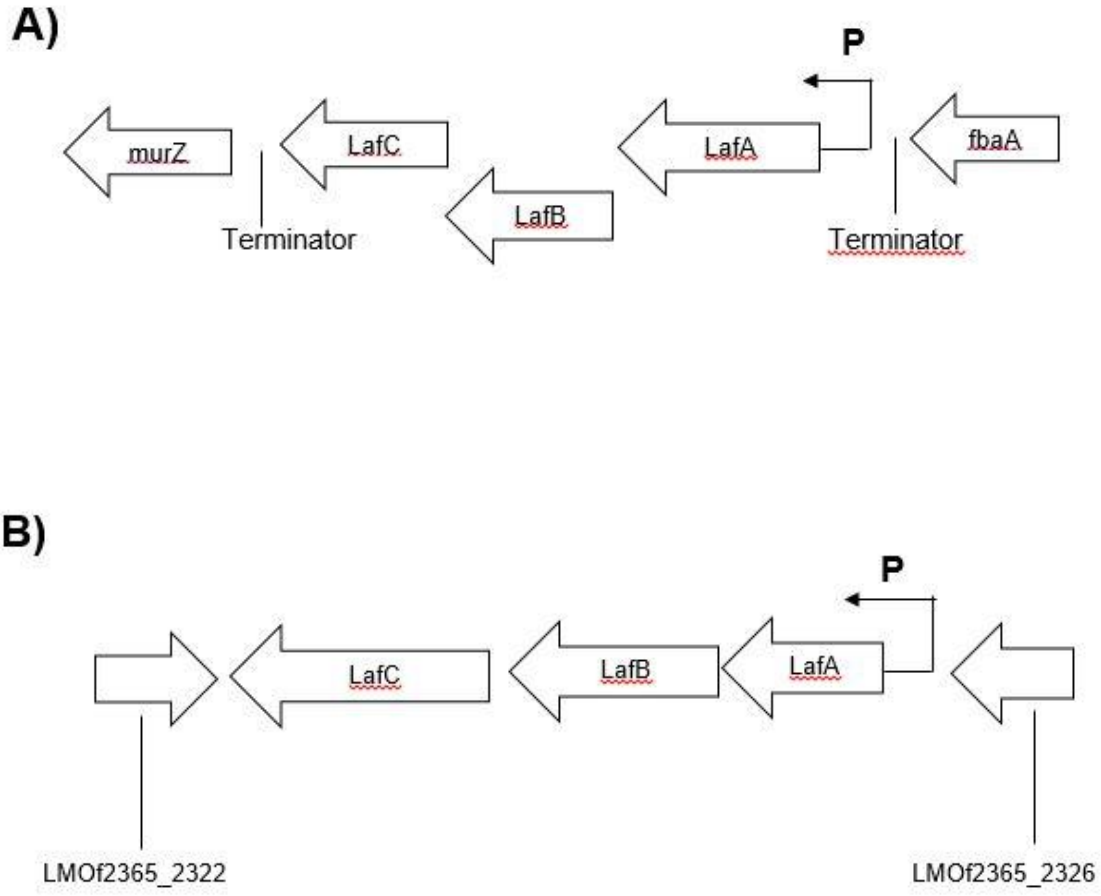


Figure 6.1. Gene map of the *lmo2555-lmo2554-lmo2553* polycistronic operon in *L. monocytogenes* strain EGD-e (A) and strain Scott A (B). The operon is designated as operon number 458 in strain EGD-e with another gene downstream of *lmo2553*, identified as *murZ* and upstream of *lmo2556*, identified as *fbaA* (181).

In all cases, transformation efficiency were also relatively low, that is in values of less than 100 CFU/µg, respectively. Previous reports have shown that the combination of the right buffer with the right methods in preparing electro competent cells likely contribute to the efficiency of transformation (170, 184-187). Other factors that contribute to electroporation efficiency are electroporation parameters (170, 184, 188, 189), cell density (184, 186, 188), and plasmid relaxation due to restriction and re-ligation of the plasmid (184). In our research, the plasmids was double restricted prior to ligation with the deletion fragments and this may be a reason behind the low number of transformants we obtained.

Following electroporation and selection at 32^{o}C, allelic exchange process starts by maintaining transformants under antibiotic selection (10 µg/mL chloramphenicol, Cm^{10}) at an elevated temperature (41^{o}C). Plasmid excision was followed by plasmid integration into the bacterial chromosome. Again, pKSV7 has been shown to excise at a much higher frequency compared to other integrative plasmids (190) in Gram positive organisms, such as *B. subtilis* (169).

Plasmid excision in *L. monocytogenes* was performed by allowing isolates to grow without antibiotic selection under permissive temperature (32^{o}C), thus allowing the temperatures sensitive origin of pKSV7 to replicate and allow for plasmid excision, generating either wild type mutant or mutant genes within the chromosome. Selection for antibiotic sensitivity was performed to confirm that plasmid has been successfully excised, and the antibiotic sensitive colonies were screened for in frame deletion mutants by PCR amplification and gel electrophoresis.

6.2 Complementation of deletion mutants

The vector pIMK2 was used for complementation. This vector has an RP4 *oriT*, and is a derivative of another plasmid pIMK, which is a kanamycin resistant site-specific integrative plasmid (170). This plasmid is based on the pPL2 vector (183), which replicates

autonomously in *E. coli* but is unable to replicate in *L. monocytogenes*, and contains a PSA listeriophage integrase gene and attachment site. Each gene fragment (*lmo2555*, *lmo2554*, and *lmo2553*) was amplified using up FWD and down REV primer pairs (CIL-*1 and CIL-*4) and ligated to pIMK2 vector. The recombinant plasmids were then electroporated into *E. coli* strain S17-1. This strain of *E. coli* has been successfully used as conjugation donor in other studies involving Gram negative organisms, such as *Flavobacterium* species (191), and Gram positive organisms, such as *Streptomyces* (192) and *L. monocytogenes* (166, 167).

Conjugation was used to mobilize each cloned complementation fragments in pIMK2 into each deletion mutant (IM55, IM54, and IM53). Conjugation donor, *E. coli* strain S17-1 was resistant to kanamycin due to the kanamycin-resistant site in pIMK2 (170), and *L. monocytogenes* is intrinsically resistant to nalidixic acid. Thus listerial transconjugants were selected using both kanamycin and nalidixic acid resistance. By conjugation, we were able to add pIMK2 plasmid into LM21 wild type strain, generating the strain IM21B. We were also able to add each gene (*lmo2555, lmo2554,* and *lmo2553*) separately into LM21, utilizing recombinant plasmids pTIR255, pTIR254, and pTIR253, generating IM255, IM254, and IM253, respectively. We observed that when either the blank vector pIMK2, or the recombinant plasmid pTIR255, pTIR254 and pTIR253 into *L. monocytogenes* wild type strain, the ability of the cell to form biofilm was not altered (results shown for LM21 and IM254 on Figure 5.6). The same phenomena were also observed when blank vector pIMK2 was inserted into each deletion mutant IM55, IM54, and IM53, generating IM55B, IM54B, and IM53B, respectively (result shown for LM21 and IM21B on Figure 5.6).

We also performed conjugation in order to complement each of our deletion mutants by introducing each recombinant plasmid, namely pTIR255, pTIR254, and pTIR253 into its respective deletion mutant strain, namely IM55, IM54, and IM53, generating strain IM55C, IM54C, and IM53C, respectively. Out of the three strains that are

generated, we were able to restore biofilm formation on strain IM54C, indicating that complementation was successful. The other two strains, namely IM55C and IM53C still showed reduced biofilm formation, where the numbers of OD measurements were comparable to those of the deletion mutants (IM55 and IM53). This indicated unsuccessful complementation of these two strains.

The unsuccessful complementation of *lmo2555* may have several possible explanations. We constructed our primers for *lmo2555* deletion based on EGD-e strain, where we designed our primers to amplify the upstream part of the gene *lmo2555*. In the EGD-e strain, *lmo2555* is directly preceded by *lmo2556* (or known as *fbaA*), which is why our PCR amplification may have amplify some of the downstream portion of the gene had we done it in EGD-e. However we used strain ScottA in our entire experiment. In ScottA, *fbaA* is not located directly upstream of *lmo2555*; it is located far upstream from it. This different location of gene preceding *lmo2555* may have contributed to the unsuccessful complementation of gene *lmo2555*.

Alternatively, the deletion may have caused polar mutation along the operon when the gene was deleted. Polar mutation may also lead to nonsense, or frame shift mutation. We eliminated the possibility of nonsense mutation because we sequenced our deletion fragments and did not find any nonsense mutation in our product. Because *lmo2555* is the first gene in the operon, it is highly possible that the deletion of this gene leads to an altered expression of the two downstream genes *lmo2554* and *lmo2553* if polar mutation takes place. If this were the case, combined with the fact that putting *lmo2555* back into the mutant did not restore the biofilm phenotype, we believe we can safely argue that biofilm formation is not merely attributed to *lmo2555* gene expression. It may be that the other two genes also play role in this aspect.

Continuing this train of thought, if it is true that the deletion of *lmo2555* affects the expression of the other two downstream genes, then it is possible to argue that deletion

of *lmo2554* maintains *lmo2555* while at the same time affecting *lmo2553*. However, bringing back *lmo2554* restored the biofilm phenotype. At this point we were not sure biofilm formation requires the expression of both *lmo2555* and *lmo2554* or just *lmo2554* and further studies need to be done in order to answer this question.

Throughout the process we were able to show that addition of integrative listerial vector pIMK2 into LM21 and each deletion mutants (IM55, IM54, and IM53) did not change their biofilm phenotype. Addition of each gene carried on pIMK2 into wild type strain also did not change their biofilm phenotype, indicating that overexpression of each particular gene did not change the ability of the strain to form biofilm on a PVC well plate on a static system. However, addition of each gene into its respective deletion strain was only able to restore the phenotype for IM54C, indicating that the *lmo2554* was complemented successfully. This led us to hypothesize that *lmo2554* may be the one gene that plays important role in biofilm formation in *L. monocytogenes.*

6.3 The influence of lipoteichoic acid in biofilm formation

Lipoteichoic acid (LTA) has been shown to act as bacterial surface antigens, and that it can have at least several numbers of determinants (20, 28). This led us to believe that immunoassay is a good way to determine the presence of LTA in our mutant strains.

We performed LTA expression assay using a dot blot immunoassay method, where we semi-quantitatively determine the presence of LTA on the surface of the cell for the wild type strain LM21, our mutant strain IM54 and IM54B, its positive control IM21B, and its complement IM54C. Based on the intensity of color formation (Figure 5.8), we were able to confirm that our mutant strain (IM54 and IM54B) produced less amount of LTA compared to the wild type strain (LM21), its positive control (IM21B), and its complement strain (IM54C). This is in agreement with previous report by Webb, *et. al.*, (144) who assess the presence of LTA in *L. monocytogenes* strain EGD-e by means of western blot

after inactivation of *lmo2555* and *lmo2554* genes. The simplest explanation for the reduction in lipoteichoic acid amount is that because the enzymes that are needed for the formation of the polyglycerol phosphate backbone, namely Lmo0927 and Lmo0644, cannot efficiently initiate lipoteichoic acid synthesis in the absence of the glycolipids (144).

In *Staphylococcus aureus*, deletion of *ypfP* gene, that is another glycosyl transferase, also leads to a reduction in LTA expression (156). The deletion of this gene did not result in a reduction in the amount of wall teichoic acid, and the growth rate of the mutant did not seem to be affected.

Even though we saw a decrease in LTA expression in our mutant strains, we did not see a difference in cell hydrophobicity (Table 5.1) on our wild type strain (LM21) and our three deletion mutants (IM55, IM54, and IM53). This is somewhat contrary to what have been reported previously. LTA so far has been known to be an amphipathic molecules, which means that they have a hydrophilic and hydrophobic region (128). In group A streptococci LTA plays a major role in conferring hydrophobicity (95, 96, 193), although other molecules may also contributes to the hydrophobicity of group A streptococci. In *Staphylococcus aureus* SA113, a significant reduction of LTA leads to a significant increase in hydrophobicity (156), which eventually lead to a significant reduction in the cells' ability to form biofilm on polystyrene and glass. This tells us that LTA is not the only component that contributes to cell hydrophobicity in *L. monoctyogenes*, thus the presence of LTA is dispensable in maintaining the level of cell hydrophobicity.

In LTA, the presence of D-alanine ester as one of its constituents contributes to a positive charge in the overall negative charge of the bacterial cell membrane. This explains how LTA might be able to maintain a high concentration of Mg^{2+} ions in the region of the membrane (70). In *Bacillus subtilis*, it has been reported that the obliteration of D-alanine ester in LTA increases the cells' affinity to a positively charged cytochrome c protein. In *Enterococcus faecalis*, the absence of D-alanine esters in LTA also increases the negative

net charge of the cell (118). This led us to believe that the absence of LTA might also lead to an increase the negative charge of the cells. However, contrary to this report, we did not see any significant difference in cell surface charge (Table 5.2) between the wild type strain LM21 and the three deletion mutants (IM55, IM54, and IM53). Even though LTA is one of the major components in the cell membrane, it is likely that when taken together as a whole system, there are other components, such as different surface proteins and ions that contribute to cell surface charge. Thus a reduction of LTA leads to a negligible effect in this particular aspect.

Previous report on transposon mutagenesis identified reduced biofilm grown with insertions in genes homologous *lmo2555* and *lmo2554* in the *L. monocytogenes* EGD-e operon (166). Others (144) were able to establish that the synthesis of glycolipid anchor in *L. monocytogenes* EGD-e consists of Gal-Glc-DAG synthesized by two glycosyl transferases encoded by Lmo2555 and Lmo2554, later referred to as Laf (LTA anchor formation) A and B respectively. LafA and LafB are thought to transfer UDP glucose and UDP-galactose to generate Glc-DAG and Gal-Glc-DAG, respectively, to generate the glycolipid anchor on which LTA is built. Since lipoteichoic acid has been shown to aid in cell adherence which contributes to an increased in bacterial virulence in streptococci (84, 85, 88), and staphylococci (90, 91, 194) we hypothesized the transposon insertions reduced levels of LTA, that then influences cell adherence and biofilm formation.

One of the key components of biofilm formation is the ability of cells to adhere to a solid surface (7, 8), and since lipoteichoic acid is one of the key components in the cell membrane, it is reasonable to assume that it has an important role in altering the ability of the cells perform such functions. Through our biofilm study using a minimal media (176) on a PVC well plate in a static system, we were able to confirm that mutant strains that are deficient of the glycolipid anchor, thus deficient in lipoteichoic acid, have severely compromised ability in biofilm formation (Figure 5.3). This is not exclusively found in *L.*

monocytogenes; previous publications have shown identical result in different Gram positive organisms, where glycosyl transferases play role in biofilm formation.

Lipoteichoic acid polymer of most Gram positive bacteria consists of D-alanine, and that the incorporation of D-alanine is regulated by series of genes in the *dlt* operon (16, 115, 116). Fabretti, *et. al.* (118) were able to confirm that the D-alanylation, or incorporation of D-alanine components in lipoteichoic acid is involved in biofilm formation, where the absence of D-alanine increases the net negative charge of the cells, thus affecting several bacterial properties, one of which is the ability of *E. faecalis* to form biofilm on a polystyrene surface.

Another publication by Theilacker *et. al.* (158) highlights the finding of two consecutive genes in *E. faecalis*, which later known as biofilm-associated glycolipid synthesis A and B (*bgs*A and *bgs*B). They were able to confirm the function of both BgsA and BgsB proteins, which are glycosyltransferase and a precursor for glycolipid and lipoteichoic acid involved in biofilm formation.

In *Staphylococcus aureus*, the protein YpfP is known as a glycolipid synthase that mediates the synthesis of DGlcDAG, where the lipoteichoic acid is anchored to (147), and that the *ypfP* gene is essential for the biosynthesis of DGlcDAG (149). Later publication by Fedtke, *et. al.* (156) confirms that even though the deletion of *ypfP* still produces lipoteichoic acid, the amount was greatly reduced, and that the mutant strain that is highly deficient of lipoteichoic acid was not able to form biofilm on polystyrene surface, thus leading them to believe that the amount of lipoteichoic acid in the cell governs the physicochemical surface properties of *Staphylococcus aureus* and enables biofilm formation.

The importance of glycosyl transferase that leads to biofilm formation has also been shown in bacilli. In *Bacillus subtilis*, the *gtaA* gene encodes the enzyme for the transfer of glucosyl group from UDP-glucose (UDP-Glc) to the polyglycerol phosphate

portion of the major wall teichoic acid (163, 164). UDP-Glc has a precursor that is isomerized from glucose-6-phosphate (Glc-6-P) by α-phosphoglucomutase, or α-PGM. The deficiency in α-PGM causes a deficiency in the UDP-Glc precursor, which is a glucosyl donor for the synthesis of phosphate-containing anionic envelope polymers in *Bacillus subtilis.* And this glucosyl group is transferred from UDP-Glc to the polyglycerolphosphate section of the major wall teichoic acid. Consequently, this all lead to a reduction in biofilm formation (162).

In order to obtain a more complete picture on how lipoteichoic acid plays role in biofilm formation, we also observed biofilm formation under flow system. Biofilm was grown for 24 hours under static condition to allow cells to adhere, before growth was prolonged for another 48 hours under flow system. In this case we performed cell counts by physically scraping cells off of a stainless steel slide instead of performing OD measurement. This should give us a better idea on the amount of living cells in the biofilm. We observed that the mutant with blank vector IM54B showed 90% lower cell counts compared to the other 3 strains (IM21B, IM254 and IM54C). Prior to that, we have determined that the three mutants IM55, IM54, and IM53 did not grow any slower than the wild type strain LM21 when grown in TSBYE and MWB (Figure 5.4). This is consistent with other Gram positive system, in this case *S. aureus*, that the absence of LTA did not alter growth rate (156). On the other hand, this may not necessarily be true for all Gram positive bacteria, since previous publications have shown that lipoteichoic acid is important in cell division and sporulation in *Bacillus subtilis* (23).

Our result so far showed that mutation in genes responsible for the synthesis of glycosyl transferases (in this case *lmo2555* and *lmo2554*) in *L. monocytogenes* lead to a reduction in biofilm formation, not only in a static system, but also in a flow system when we grew our biofilm using a drip flow reactor (Figure 5.9), where our mutant strain IM54B gave a lower cell number compared to the other three strains. Since we have eliminated

the possibility of a lower growth rate contributing to the lower cell counts we observed in our biofilm, we started looking into other possibilities of how the absence or a significantly lower amount of lipoteichoic acid in the cell contributes to a reduction in cell counts.

In order to assess how lipoteichoic acid influences biofilm growth on glass slide, we grew our biofilm using a drip flow reactor and visualize the cells within the biofilm community using FM 1-43 green biofilm cell stain prior to observation under confocal microscope. FM dyes are lipophilic styryl compounds used in a wide variety of studies involving plasma membrane and vesiculation. The dye is water-soluble and virtually non-fluorescent in aqueous media, and are believed to insert into the surface membrane where they become intensely fluorescent. FM 1-43 stain has been used extensively to stain the cell bodies specifically in a complex biofilm system, including *P. aeruginosa*, *E. coli*, *Staphylococcus* sp., and *V. cholera*.

The confocal microscopy images showed thicker biofilm community for our IM21B strain, and a lot thinner and sparser cells for our IM54B mutant (Figure 5.10). Since the FM dyes are lipophilic compounds, it is very likely that it stains the hydrophobic portion within the cell membrane. The complement strain IM54C, however, showed comparable cell density compared to the mutant strain IM54B. This is a confirmation that deleting *lmo2554* decreases cell density in biofilm community, and that bringing back the gene restores cell density, making it comparable to the wild type.

Up to this point we are fairly confident that the lower cell counts in our biofilm are most likely due to the fact that the absence (or the significantly reduced amount) of lipoteichoic acid in our mutant strains decreases the cell's ability to adhere to a stainless steel slide and not because of the lower growth rate. However, this may not necessarily be true for all Gram positive bacteria.

Theilacker, *et. al*. (150, 158) were able to show that the impaired biofilm formation in *Enterococcus faecalis* is more likely due to the defective accumulation of biofilm mass

after initial attachment. Over a period of 24 hours, biofilm mass of the wild type strain on polystyrene grew in a linear fashion, while the amount of biofilm produced by *bgsA* and *bgsB* mutants remained constant at the level of initial attachment.

In order to confirm our hypothesis, we performed an initial attachment study with our three mutant strains, that is between LM21 and IM55, IM54, and IM53 (Figure 5.5) and saw a difference in initial attachment between LM21 and both IM55 and IM54 after 30 minutes, 2 hours and 5 hours. However, we did not see a difference in initial attachment between LM21 and IM53, which means that the deletion of *lmo2553* did not alter the cells' initial attachment to a PVC plate.

Because we saw a difference between the wild type strain and the two mutant strains in initial attachment, we performed another set of initial attachment assay after complementation using IM54B mutant strain with its two positive controls (IM21B and IM254) and its complement strain IM54C. We saw that the complementation restored the cells' initial attachment ability at all time points. All this combined, we believe it is a safe assumption that the reduction in biofilm formation in our mutant strain is due to the cells' initial attachment ability, and not because of the slower growth rate.

Previous publication (144) has confirmed that the glycosyl transferases are responsible for the formation of glycolipid backbone Gal-Glc-DAG of the lipoteichoic acid, and that the absence of the glycolipid backbone lead to a significant reduction in the amount of lipoteichoic acid in the cell. We observed similar phenomena when we did LTA expression analysis (Figure 5.8). We were able to confirm that our mutant strains IM54 and IM54B express less amount of LTA based on their less intense color compared to the two positive controls (LM21 and IM21B), and the complement strain (IM54C). We observed similar trends, that is, our mutant strain IM54 and IM54B showed reduced biofilm formation ability compared to the wild type strain LM21, its positive controls IM21B and IM254 when grown on different surfaces (PVC, stainless steel, and glass), both in stagnant

and flowing conditions. We were able to show this phenomena quantitatively, both with OD measurement (Figure 5.3, and 5.6), and cell counts (Figure 5.9), and qualitatively with confocal microscopy (Figure 5.10). Altogether, this leads us to believe that lipoteichoic acid plays an important role in *L. monocytogenes* biofilm formation. This is in agreement with previous publications that the importance of these glycosyl transferases is not merely observable in *L. monocytogenes*, but also in other Gram positive bacteria, such as *E. faecalis* (150, 158), *S. aureus* (149, 156), and *B. subtilis* (162).

6.4 The influence of lipoteichoic acid (LTA) on the survival of *L. monocytogenes* in the presence of different antimicrobials

We have shown that the deletion of *lafB* gene in *L. monocytogenes* strain ScottA leads to a reduction in lipoteichoic acid (Figure 5.8). This is similar to what have been observed by Webb, *et. al.* (144) in *L. monocytogenes* strain 10403S. Lipoteichoic acid has been shown as a major constituent in Gram positive organisms, and that its presence affects cell surface properties (128).

We evaluated how lipoteichoic acid influences the resistance of *L. monocytogenes* to benzethonium chloride (BZT) and cetylpyridium chloride (CPC), two quaternary ammonium compound sanitizing agents. We found almost no difference in MIC between the mutant strain IM54B, its two positive controls IM21B and IM254, and its complement strain IM54C (Table 5.3). Bacterial cell surface is largely negatively charged. Previous reports have noted the importance of D-alanine components in lowering the net negative charge in the bacterial cell wall (118, 195). BZT and CPC are non-charged molecules, thus their mechanisms may not be affected as much by the surface charge of the cell. This explains why the absence of LTA did not seem to influence the cells' ability to tolerate quarternary ammonium compounds.

We also selected charged antimicrobials: cationic Lauric arginate (LAE) and anionic sodium dodecyl sulfate (SDS). Mutant strain IM54B is significantly more sensitive to both LAE and SDS compared to its positive controls IM21B and IM254, and its complement strain IM54C. LAE is a cationic surfactant that has antimicrobial activity. It has been shown that the esterification of D-alanine in LTA increases the positive charge of the cell envelope through the additional amine groups. Therefore, the hypothesis so far is that the absence of D-alanine ester leads to a reduction in the net negative charge, thus making the cells more resistant to cationic antimicrobials (195). A similar phenomena has also been reported in other Gram positive systems, such as *Enterococcus faecalis* (118), *Staphylococcus aureus* (112), and *Streptococcus pneumoniae* (196).

In our system, we did not observe any difference in measured cell surface charge between our wild type strain LM21 and our three deletion mutants IM55, IM54, and IM53 (Table 5.2). In addition to that, we observed similar trends with both anionic and cationic antimicrobials, where the mutant strain is more sensitive compared to the two positive controls and the complement strain. Taken together, this strongly suggests that an increase in sensitivity to LAE and SDS is not due to the effect of LTA has on cell surface charge.

We believe that the increased sensitivity of our mutant strain compared to its positive controls and its complement strain may be due to other mechanisms and may not have anything to do with how the LTA affects the surface charge of the cell. Due to its polyanionic character, LTA bind cations rather effectively, where divalent cations are bound more effectively than monovalent cations (17, 50). Of all divalent cations required for bacteria to maintain growth, magnesium ions are known to be required for the stability of cell membranes and the activity of many membrane-bound enzymes. It has been suggested that one major functions of LTA is to maintain a high concentration of magnesium ions in the membrane (70). In *Bacillus licheneformis*, magnesium-dependent

enzymes in the cytoplasmic membrane can only be activated when magnesium ions are bound to LTA (71). In staphylococci, LTA has also been shown to regulate the surface charge and cation binding action of the cell wall. This leads us to believe that the absence of LTA significant increase in sensitivity to both LAE and SDS may have been due inability of membrane enzymes to function properly without the presence of cations bound by LTA, thus compromising the integrity of the membrane.

Another aspect that may play role in the increased sensitivity of our mutant strains against antimicrobials is autolysin activity. Autolysin enzymes in Gram positive organisms cleaves the peptidoglycan strands to separate daughter cells upon cell division. However, an increased activity of autolysin may lead to a degradation of cell membrane which lead to cell death. Autolysin enzymes also have high affinities for LTA and have been proposed to be positioned in cell wall septa by cell wall glycopolymers (59). Peschel, *et. al*. (112) have shown that autolysin activity in *Staphylococcus aureus* is highly regulated and strictly controlled by the binding of cationic autolysin to the anionic portion of LTA. Looking at it from this point of view, the absence of LTA might lead to an increase in autolysin activity, which eventually lead to an excessive degradation of the bacterial cell membrane, thus increasing the cells' resistance toward antimicrobials.

6.5 The influence of lipoteichoic acid (LTA) on the growth of *L. monocytogenes* at low temperature and high osmotic stress

In order to assess the influence of lipoteichoic acid on the survival of *L. monocytogenes* under environmental stress, we grew our mutant strain IM54B along with our positive control (IM21B) and its complement strain IM54C on TSAYE plates with different water activities (a_w) and incubate them at different temperatures. Because previous results have shown different humectants have different effects on the survival of

L. monocytogenes (134) and other Gram positive systems (197), we used three different humectant to lower the a_w of our plates, namely NaCl, sucrose and glycerol.

Our result showed that lower temperature decreases growth rate in all three strains (Figure 5.11). The influence of lipoteichoic acid was more pronounced at low temperature (4^{o}C), but not as much at higher temperature, where LTA-deficient mutant did not exhibit slower growth when incubated at 32^{o}C and 37^{o}C. This led us to believe that lipoteichoic acid is indispensable for the growth of *L. monocytogenes* at low temperature (4^{o}C) while not as much at higher temperature (32^{o}C and 37^{o}C). However, contrary to our findings, previous result by Oku, *et. al.* (129) showed that lipoteichoic acid is dispensable in *Staphylococcus aureus* at permissive temperature (30^{o}C), while not at higher temperature (37^{o}C and 43^{o}C). They stated that lipoteichoic acid is essential for colony formation, cell viability and resistance to low osmolarity condition at higher temperature.

It has been shown in previous publications that in *L. monocytogenes*, there is a close relationship between cell morphology and temperature. Rowan, *et. al.* (198) showed that thermotolerance, or heat resistance in *L. monocytogenes* is influenced by cell morphology, where they saw that cutures that have more long chain formation are more thermotolerant than the ones that do not have long chain formation. Thus they concluded that one of the factors that influence the influence of thermoresistance of suspended *Listeria* cells is cell morphology. Another publication by Zaika, *et. al.* (199) also stated that temperature was a major factor in certain stress conditions that led to cell elongation and loss of flagella in *L. monocytogenes*.

In order to test how growth temperature affect the growth of our strains, we grew our wild type LM21, its positive control IM21B, our mutant strains IM54 and IM54B, along with its complement strain IM54C in TSBYE at different temperatures; 4^{o}C, 20^{o}C, 32^{o}C, and 37^{o}C. We visualize the cells under the microscope with a DIC filter and we did not see a significant difference in cell morphology (results not shown).

LTA has been known to act as divalent cations (17, 50) and helps to maintain high concentration of magnesium ions in the membrane where magnesium ions are required to maintain the stability of the cell membrane (70), and that in *Bacillus licheniformis* magnesium-dependent enzymes can only be activated by magnesium when the magnesium is bound to LTA. The absence of LTA limits the amount of magnesium bound, thus compromising the integrity of the cell membrane. Another explanation might be due to the amount of LTA present. Dehus *et. al* (145) has shown that *L. monocytogenes* expresses two different variants of LTA, where the amount of one particular LTA (known as LTA2) is significantly lower when cells are grown at lower temperature. This might also be the reason why our LTA-deficient mutant strain IM54B cannot grow at 4^{o}C as well as its positive control IM21B and IM54C. However, we cannot be sure of this because we did not see any noticeable difference in our dot blot experiment when we grew the cells at 20^{o}C and 37^{o}C (results not shown). It is important to note, of course, that our semi quantitative detection method may not be sensitive enough to show the different amount of LTA when *L. monocytogenes* are grown at different temperatures.

At 20^{o}C however, it seemed that the mutant strain IM54B grew slower than its positive control IM21B and its complement strain IM54C. This might be caused by a slightly lower initial inoculation per drop (Figure 5.11)

We also found that our mutant strain IM54B exhibits different sensitivities to different humectant at different a_w, although the trend seems to be similar across different temperatures. At 4^{o}C (Figure 5.12), the mutant strain did not show any difference in sensitivities compared to its positive control IM21B and its complement IM54C in the presence of NaCl before the a_w was lowered to 0.95, which means that low concentration of NaCl seem to recover the ability of our mutant strain to tolerate low temperature.

Similar observations have also been made by Oku, *et. al.*, where higher osmolarity seems to be required in order to recover growth in LTA-deficient mutant in *S. aureus* (129)

LTA contains anionic polymers of phosphoglycerol, and thus releases fixed anions on the outside of the cell membrane. LTA and its counter ions could provide local osmotic pressure on the outside of the cell membrane and could eventually reduce osmotic stress. They argued that this is the reason why high osmolarity condition can recover the growth of *S. aureus* that is LTA-deficient. What we saw in our LTA-deficient *L. monocytogenes*, however, is that low concentration of NaCl (4.37% at a_w 0.98) can recover growth in our LTA-deficient mutant at low temperature, while higher NaCl concentration reduced cell survival. This might be due to the different LTA composition between *S. aureus* and *L. monocytogenes* which leads to a different fixed anions released in the outside of the cell membrane. This eventually will lead to a different level of osmolarity required to recover the growth of LTA-deficient mutants.

Since high osmolarity and low temperature are conditions that favor *L. monocytogenes* over its competitors, the processes of osmotic adaptation and low temperature adaptation are crucial to its importance as a foodborne pathogen. One mechanism of osmotic stress adaptation commonly found in bacteria involves intracellular accumulation of organic compounds called osmolytes (137-139) which contribute to a counterbalancing osmotic pressure, and one of the most ubiquitous and effective os one called glycine betaine (139). It has been shown in previous reports that the concentration of glycine betaine as a cryoprotectant and osmoprotectant increases significantly in *L. monocytogenes* when grown in low temperature, and even more so in the presence of 8% NaCl (131). This means that NaCl enhances glycine betaine transport which causes an increase its concentration intracellularly, thus increasing the tolerance of cells to low temperature.

The osmolyte transporters BetL, Gbu, and OpuC were also induced upon cold shock in *L. monocytogenes* LO28, although in different degrees (141). These might explain the growth recovery of our LTA-deficient mutant at 4^{o}C accompanied by low

concentration of NaCl (a_w 0.98 or 4.37% NaCl). And because the presence of other humectant did not seem to recover the growth of our LTA-deficient mutant, it is highly possible that the recovery effect is exclusively found in NaCl and not in other humectants.

When a_w was lowered to below 0.93 and 0.90, however, our mutant strain seem to not be able to tolerate low temperature. This might not due to the presence or absence of LTA, but simply because the a_w was lowered to the point where the osmotic stress was far too much for the cells to withstand, even in the presence of LTA. However, it is important to note that LTA did not seem to affect the survival of *L. monocytogenes* in the presence of NaCl at higher temperature (Figure 5.13, 5.14, and 5.15).

Low salt concentration has been shown to improve survival of *L. monocytogenes* at limiting pH value, where higher salt concentration reduced their survival (133). What we saw in our result might tell us that low osmolarity medium improve cell survival not only at their limiting pH value, but also at their limiting growth temperature. *L. monocytogenes*, although classified as mesophiles, are still metabolically active at temperature as low as 5^{o}C, as reviewed by Bereksi, *et. al.* (130), and that these adaptation seems to be very temperature-dependent (133). Survival of *L. monocytogenes* under extreme conditions such as low temperature and how they can stay metabolically active at low temperature has been shown to be related to different things, such as morphological features (200) and modification in the composition of surface protein (201).

Among the three humectants used, glycerol seemed to be the less harsh to all strains. Even at lower temperature (4^{o}C), when glycerol was used as humectant, growth was still visible at a_w 0.93, while growth was not visible on plates when NaCl or sucrose was used. At higher temperature, the sensitivity of mutants did not seem to be different from its positive control IM21B and its complement strain IM54C at all a_w levels, except when a_w was lowered to 0.93 and incubated at 37^{o}C. This may be because glycerol is known to be membrane permeant, thus it does not cause osmotic stress to the cells (135).

Kempf, *et. al*. (202) have also stated in their review that glycerol is a highly soluble molecules and do not carry a net charge at physiological pH, thus can reach high intracellular concentration without disturbing vital cellular functions.

Sucrose, like glycerol, is also a highly soluble molecule that does not carry a net charge at physiological pH (202), thus explaining why the positive control IM21B and IM54C can still grow in the presence of sucrose at higher temperatures (20^{o}C, 32^{o}C, and 37^{o}C) even when a_w is lowered to 0.93. Our mutant strain, however, showed different sensitivity to sucrose, where its growth was severely compromised at higher temperatures when a_w was lowered to 0.95 and below, thus showing the importance of LTA in the survival of *L. monocytogenes* in high osmolarity medium due to the presence of sucrose. This can also be explained and tied back to the role of LTA as osmoprotectants and its similarities to osmoregulated periplasmic glycans (OPGs) in Gram negative organisms (126). Bhagwat, *et. al*. (127) also have explained how OPGs accumulate in Gram negative periplasm and are thought to protect bacteria under these conditions. The absence of LTA devoids the cells of its osmoprotection, thus compromising the cells' survival in high osmolarity medium.

CHAPTER VII

CONCLUSION

- Lipoteichoic acid (LTA) in *L. monocytogenes* is essential for biofilm formation on PVC, stainless steel and glass, both in stagnant and flow condition.
- Loss of LTA did not alter cell growth, hydrophobicity and surface charge.
- Loss of LTA alters the ability of cell to perform initial attachment to a PVC surface.
- Loss of LTA increased the sensitivity of *L. monocytogenes* to anionic, cationic, and quarternary ammonium compounds (QAC) antimicrobials.
- LTA is important for *L. monocytogenes* growth in low temperature.
- The presence of sodium chloride (NaCl) at low levels (a_w 0.98) enhanced survival of LTA-deficient *L. monocytogenes* at 4^{o}C.
- LTA is essential to counteract the effect of high osmolarity in the presence of sucrose, and to a less extent with NaCl and glycerol.
- Further understanding of biofilm formation mechanism will eventually lead to a more intelligent design of *L. monocytogenes* biofilm removal in the food processing environment.

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